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Hazard Evaluation: Human and Domestic Animals

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SUBDIVISION F

HAZARD EVALUATION:

HUMAN AND DOMESTIC ANIMALS

by

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As a guideline for the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Subdivision F supports the data requirements of 40 CFR Part 158. Subdivision F describes protocols for evaluating the toxicity of pesticides to nonhuman organisms and for relating the results of these studies to human safety evaulations. Comments from environmental groups, academia, and industrial representatives were incorporated into the current draft.

Subdivision F constitutes one volume of a twelve-part FIFRA guidelines series published by the National Technical Information Service.

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Foreword

Toxicity data related to human and domestic animal safety evaluation are required to support pesticide registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Subdivision F describes study protocols which may be used to develop these toxicity data. Public comment on Subdivision F was accepted in a series of public meetings, the last of which was held in July 1982. Because of the importance of the issues raised in the Subdivision F comments, Subdivision F was redrafted with the help of a review committee comprised of representatives from industry, environmental groups and academia. This guideline refers to requirements established in 40 CFR Part 158 so that this document can be read as a complete package and so that the protocols may be explained in their proper context.

SUBDIVISION F: HAZARD EVALUATION: HUMANS AND DOMESTIC ANIMALS

Table of Contents

DISCUSSION

PAGE

I. Organization and Philosophy of Subdivision F 1

II. The Standards for Acceptable Testing 3

III. Discussion of Individual Tests 12

- A. Acute Oral, Dermal, and Inhalation Toxicity 12
- B. Irritation and Sensitization Studies 13
- C. Neurotoxicity Evaluations 13
- D. Subchronic Studies 14
- E. Chronic and Oncogenicity Studies 15
- F. Teratogenicity and Reproduction Studies 16
- G. Mutagenicity Studies 17
- H. Special Testing 18

GUIDELINES

Series 80 Overview, Definition, and General Requirements

- 80-1 Overview 20
- 80-2 Definitions 20
- 80-3 General Provisions 21
- 80-4 Reporting of Data 28
- 80-5 Combined Testing 32

Series 81 Acute Toxicity and Irritation Studies

- 81-1 Acute Oral Toxicity Study 34
- 81-2 Acute Dermal Toxicity Study 39
- 81-3 Acute Inhalation Toxicity Study 44
- 81-4 Primary Eye Irritation Study 51
- 81-5 Primary Dermal Irritation Study 55
- 81-6 Dermal Sensitization Study 59
- 81-7 Acute Delayed Neuerotoxicity of Organophosphorus Substances 62

Series 82 Subchronic Testing

- 82-1 Subchronic Oral Toxicity (Rodent and Non-rodent): 90-Day Study 66

Table of Contents (Continued)

GUIDELINES		PAGE
82-2	Repeated Dose Dermal Toxicity: 21-Day Study	76
82-3	Subchronic Dermal Toxicity: 90-Day Study	83
82-4	Subchronic Inhalation Toxicity: 90-Day Study	92
82-5	Subchronic Neurotoxicity: 90-Day Study	103
Series 83	Chronic and Long Term Studies	
83-1	Chronic Toxicity Studies	107
83-2	Oncogenicity Study	117
83-3	Teratogenicity Study	125
83-4	Reproductive and Fertility Effects	130
83-5	Combined Chronic Toxicity/Oncogenicity Studies	137
Series 84	Mutagenicity	
84-1	Purpose and General Recommendations for Mutagenicity Testing	147
84-2	Mutagenicity Tests	148
Series 85	Special Studies	
85-1	Metabolism Study	152
85-2	Domestic Animal Safety Testing	156
85-3	Dermal Absorption Studies of Pesticides [Reserved]	

I. ORGANIZATION AND PHILOSOPHY OF SUBDIVISION F

[NOTE: The following Discussion constitutes a reiteration of many parts of the preamble to the Subpart F guidelines proposed on Aug. 22, 1978 (43 FR 37336). Much of that information, instruction, and explanation apply to the current guidelines. Minor changes have been made to update some of the preamble text to apply to the current guidelines. Most of the preamble discussion material included in the 1978 preamble was deleted in the current discussion.

General requirements and definitions which apply to many or all of the sections in Subdvision F appear in the Overview and related sections, in the § 80 series. For the rest of this subdivision, each kind of test is described in a separate section of the guidelines. Similar tests are grouped together in a series. The short term acute tests appear in the § 81 series; the subchronic tests appear in the § 82 series; and the long term, chronic tests appear in the § 83 series. In addition, the § 84 series contains tests for evaluating mutagenic effects, and the § 85 series contains special kinds of tests.]

A. General Information and Requirements.

1. Proposed rule, 40 CFR Part 158, specifies the kind of data and information that must be submitted to EPA to support the registration of each pesticide under the Federal Insecticide, Fungicide and Rodenticide Act. The Agency intends to promulgate Part 158 as a final rule during 1983. This subdivision provides detailed information relating to the data requirements listed in 40 CFR Part 158 including the conditions under which each data requirement is applicable, the standards for acceptable testing, stated with as much specificity as the current scientific disciplines can provide, and the information that should be included in a test report.

2. Data requirements for manufacturing-use products. In the Preamble to the 1978 proposed Guidelines, EPA asked for public comment on the question whether the data requirements of this subdivision should be extended to manufacturing-use products. After serious consideration of this issue, the Agency has concluded that extending the data requirements to such pesticides is appropriate. The Agency was influenced by the views of commenters on this issue who generally favored a data submission requirement which makes the basic manufacturer of an active ingredient responsible for providing most of the environmental fate data.

Therefore, a section of 40 CFR Part 158, entitled "Formulators' Exemption" (§ 158.50), requires a registrant of a manufacturing-use product to submit (or cite) any data pertaining to the safety of an active ingredient in its product if the same data are required to support the registration of an end-use product that could legally be produced from the registrant's manufacturing-use products. (An end-use product is a pesticide product bearing label directions for immediate end-use as a pesticide.) Section 158.50 also provides that such data must be submitted by an applicant for registration of end-use product, except that the producer of the end-use product will generally not have to submit or cite data pertaining to registered products which the end-use producer purchases and uses to formulate the end-use product. This decision reflects the Agency's expectation that manufacturing-use product registrants will be the major source of registration data, and that end-use product formulators will, in most cases, need to supply much less data. This decision is consistent with the provisions of, and Congressional intent behind, § 3(c)(2)(D) of FIFRA, which provides that:

> No applicant for registration of a pesticide who proposes to purchase a registered pesticide from another producer in order to formulate such purchased pesticide into an end-use product shall be required to--
>
> (i) submit or cite data pertaining to the safety of such purchased product; or
>
> (ii) offer to pay reasonable compensation otherwise required by § 3(c)(1)(D) of FIFRA for use of any such data.

Implicit in § 3(c)(2)(D) is Congress' expectation that it would be the registrant of the manufacturing-use product who would provide significant amounts of data pertaining to the safety of its product. (See, e.g., Sen. Rep. No. 334, 95th Cong., 1st Sess., pp. 8-9.)

Moreover, if data requirements were imposed solely on registrants of end-use products, § 3(c)(2)(D) might be read to prevent the Agency from obtaining data on the grounds that the data pertain to the safety of a purchased product.

B. "Combined testing" Paragraphs.

Since EPA is concerned with efficient use of test animals, laboratory facilities, and personnel resources, the Agency encourages applicants and registrants to combine two or more different assays into a single test. In individual sections (See e.g. §§ 82-1 through -5 and §§ 83-1, -2 and -5) assay systems are identified which could be combined, provided, of course, that the principles for both types of testing are met. In addition to the specific identified combinations, proposed § 80-5 states that data required by this subdivision may be derived from test methodologies which satisfy the principles for acceptable testing contained in two or more different sections of this subdivision.

The Agency considers that published protocols which cover more than one assay system would be useful to some applicants and registrants. Accordingly, as such protocols are developed, EPA will add them to the lists of references in the appendices which appear at the ends of the test sections.

C. "Data reporting and evaluation" Paragraphs.

A paragraph describing the data reporting requirements follows the "Principles" paragraph in each of the individual test sections. The requirements in these paragraphs, together with the requirements in § 80-4, would prescribe the format and content of test data reports. Generally, the test report shall include a summary and evaluation of the test results, and specific detailed information to support the conclusions presented in the summary.

The paragraphs specifying the data which shall be reported are quite detailed and ask for considerable basic information. This information is necessary, however, to permit Agency staff to make their own independent analysis of a test and to check the accuracy of an applicant's analysis. In addition, the paragraphs recommend applicants to submit more analysis and evaluation of data than before. EPA considers it appropriate for applicants, in addition to Agency staff, to perform this work.

II. THE STANDARDS FOR ACCEPTABLE TESTING

This part of the Discussion explains certain issues which have arisen with respect to the standards governing the methodologies for performing tests. The issues discussed here concern standards which would apply to the performance of several kinds of tests. Issues involving technical aspects of the standards which appear in only one kind of test are discussed in part III of this Discussion.

A. Test Substance.

The "when required" paragraphs of the guidelines describe which products would have to be supported by data. The "test substance" paragraphs prescribe what compound would have to be tested in order to generate the required data. In many instances, data required to support the registration of the pesticide product would be derived from tests performed with a substance which is a component of the product itself.

The "test substance" paragraphs usually would require testing with some combination of the following three substances: the technical grade of each active ingredient in the product, the manufacturing-use product, or the end-use product. "Technical grade of the active ingredient" or "technical chemical" refers to a particular chemical substance, the active ingredient, having a certain level of purity, the technical grade. Presently, the purity of the technical chemical is not fixed by an absolute standard. It is usually determined instead by traditional industry methods, and usually refers to that identifiable stable substance produced during the normal manufacturing process which contains the highest concentration of the active ingredient.

In all tests, the composition of the test substance would have to be precisely identified. [See § 80-3(b)(2)(iv).] When the required test substance is a manufacturing-use product or end-use product, its composition should meet or approximate the limits certified in accordance with § 62-2 of the Subdivision D product chemistry guidelines. In those cases when the test substance does not conform with these limits, the Agency shall make a determination as to the toxicological significance of the deviations. The purposes and uses of certified limits are discussed in more depth in the Discussion on Subdivision D.

The lists below summarize the substances which would usually be tested to satisfy the different data requirements:

Technical chemical:

- Acute oral toxicity - § 81-1
- Acute dermal toxicity - § 81-2
- Acute inhalation toxicity - § 81-3
- Acute delayed neurotoxicity - § 81-7
- Subchronic oral toxicity - § 82-1
- Subchronic dermal toxicity - § 82-2, -3
- Subchronic inhalation toxicity - § 82-4
- Subchronic neurotoxicity - § 82-5
- Chronic toxicity studies - § 83-1
- Oncogenicity - § 83-2
- Teratogenicity - § 83-3
- Reproduction - § 83-4
- Mutagenicity - § 84-1 and 2
- Special Studies - § 85-1 and 2 - An analytically pure grade of active ingredient

Manufacturing-use product:

- Acute oral toxicity - § 81-1
- Acute dermal toxicity - § 81-2
- Acute inhalation toxicity - § 81-3
- Primary eye irritation - § 81-4
- Primary dermal irritation - § 81-5
- Dermal sensitization - § 81-6

End-use product:

1/ Acute oral toxicity - § 81-1
1/ Acute dermal toxicity - § 81-2
Acute inhalation toxicity - § 81-3
Primary eye irritation - § 81-4
Primary dermal irritation - § 81-5
Dermal sensitization - § 81-6

In many cases a registered manufacturing-use product would be a technical chemical. When this is the case, a single acute oral study using the product would, for example, satisfy the requirements to test both the technical grade of the active ingredient and the manufacturing-use product. However, when the manufacturing-use product is not a technical chemical, separate studies with the product and the technical chemical would be required.

When test data would be used primarily as the basis for requiring label warnings or special packaging, the Agency would require the product itself to be tested. (In some cases, EPA would also require tests on use dilutions.) Thus, all of the studies in Series 81 (acute toxicity and irritation testing), except acute delayed neurotoxicity, 2/ would be performed with the product for which registration is sought, either the manufacturing-use product or the end-use product.

The Agency uses data from acute studies on the technical grade of the active ingredients to establish the relative toxicity of the chemicals, to identify possible synergistic agents, and to evaluate the design of subchronic tests. EPA considers the data on acute oral and acute dermal LD50, and acute inhalation LC50, to be basic toxicological data from which to begin the evaluation of any chemical.

The majority of the other tests in this subdivision are subchronic and chronic studies, and the guidelines generally require that they be performed with the technical grade of each active ingredient in the product. The decision to routinely require intermediate and long term toxicity testing only on technical chemicals is based both on the Agency's experience in reviewing the toxicity of the ingredients of pesticide products and on economic considerations.

1/ Use dilutions of the end-use product are tested in addition to the formulation.

2/ The acute delayed neurotoxicity study differs from the other acute studies in that the Agency would rarely impose the usual label warnings or special packaging requirements as a result of data from this study alone. Rather, this study is used as a screen, and if positive results occur, further testing is required. (See § 82-5.) Thus, acute testing for delayed neurotoxicity is required routinely only for certain technical chemicals.

Pesticide products contain many different kinds of ingredients. Every pesticide contains at least one pesticidally "active ingredient," which by definition produces toxic or behavior-altering effects in one or more kinds of organisms exposed to the chemical. The ordinary commercial production of pesticidally-active ingredients usually introduces manufacturing impurities or byproducts which may also be toxic. Other chemical substances called "inert ingredients" are defined by FIFRA 3/ as any ingredient in a product which is pesticidally inactive. These substances, however, are not necessarily non-toxic, nor are they necessarily biologically or chemically inert. Some inert ingredients may be added to improve the usefulness of the pesticide, to reduce its hazard to users, to dilute the chemicals, to stabilize the ingredients, and for several other reasons. An "inert ingredient" may bring its own impurities or may react with other ingredients or packaging materials to produce new impurities. Finally, some pesticides are somewhat unstable and over time may partially degrade to form still more substances.

EPA recognizes that any of the ingredients in a pesticide product could be toxic to man or other beneficial organisms. Experience, however, has shown that, with some notable exceptions, the active ingredients and associated manufacturing impurities are of greatest concern. These impurities, together with the active ingredient, make up what is usually considered the "technical chemical." Thus, testing the technical chemical is consistent with that experience.

B. Number of Animals.

Each individual test section would specify a minimum number of animals to be tested. The decisions on numbers of animals were based on the recommendations of experienced Agency and OECD scientists. Their recommendations, in turn, were based on a number of factors, including their knowledge of the incidence of unusual and significant toxicological and pharmacological effects, the level of statistical and technical confidence that each kind of test should have for regulatory decision-making, and the relative cost of larger and smaller numbers of animals. The Agency recognizes that increasing the number of animals in any test may improve the sensitivity of the test. But an increase would also raise the cost of the test. EPA considers the number of animals to be a reasonable accommodation to the competing interests in providing adequate sensitivity for the test systems and minimizing costs.

The statistical significance of test results is very important for Agency decision-making. This is handled in two ways in the guidelines. In the case of acute oral, dermal, and inhalation studies, the standards would require that enough animals be used so that the LD50 or LC50 of the test

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3/ See FIFRA Sec. 2(m)

substance can be determined with a specified level of statistical certainty. This approach is reasonable for these acute studies which are designed to quantify a single toxic effect. It would be inappropriate for other tests in which the Agency is concerned with a number of different toxic and pharmacological effects. In addition, the general principles governing all testing provide that, if a toxicological or pharmacological effect occurs with only marginal statistical significance, EPA may require that the test be performed again with a larger number of animals. [See § 80-3(b)(10)].

C. Range-Finding Studies and Selection of Dose Levels.

The principle concerning selection of dose levels generally specify that the chosen levels would have to produce a particular result. For example, § 83-3 would recommend that the highest of the three dose levels in a teratogenicity study must produce some toxic effect in the treated mothers.

The recommendation that the highest dose level would have to produce some toxic or pharmacological effect appears in several sections, and is designed to ensure that the test is performed at a sensitive level. (However, EPA may accept data from a study which appears valid even when an extremely high dosage level fails to elicit a toxic response.)

Each subchronic section recommends that the highest dose level be chosen to result in definite but not excessive toxicity. The Agency feels that the high dose level should be chosen to characterize the maximally tolerated dose rate for these exposure periods in order to allow sufficient spread between the lowest and intermediate dosage ranges to obtain good dose-response information.

A number of sections also contain a principle that the lowest dose level should produce no evidence of toxicity. The requirement for a "no observed effect level" comes primarily from EPA's tolerance-setting procedures under the Federal Food, Drug, and Cosmetic Act (21 USC 321 et seq.) 4/. To set a tolerance with an ample margin of safety, the Agency calculates an acceptable daily intake by dividing the "no observed effect level" for a pesticide by an appropriate safety factor which depends upon the type of study and the toxic effect observed. Residues of that pesticide in food or feed up to the tolerance level are permissible. Similar considerations affect the Agency's decisions to approve certain other kinds of use patterns for pesticides.

4/ This Discussion describes the Agency's tolerance testing process only briefly. A more comprehensive explanation of the mechanics of establishing tolerances can be found in EPA's "Tolerance Paper" which may be requested from EPA. See also part XIV of the Discussion on Subdivision M for the FDA 1968 guidelines on residue tolerances.

As noted above, EPA ordinarily uses the no observed effect levels established in various studies in making regulatory decisions concerning safe levels of human exposure. Some use patterns, however, may involve accidental inadvertent human exposure at levels higher than the no observed effect level. To evaluate risk in these situations, EPA would need data from dose levels substantially higher than the anticipated level of human exposure. If the dose levels of an available study would not give the Agency adequate information to approve a particular use, EPA would require that additional testing be conducted.

The Agency recognizes that in order to satisfy these principles, applicants may find it useful to perform a range-finding test before beginning the full scale study. The Agency also recognizes that these preliminary tests, which generally use fewer animals and more dose levels than required by the current guidelines, can yield other valuable information helpful to the design of the full scale study. These guidelines, however, would not require applicants to perform this preliminary testing, both because such tests are not always needed and because the appropriate design for a preliminary test depends on what information is already known. Consequently, it would be difficult to specify in the guidelines when and how range-finding studies should be conducted.

D. Control Groups.

Most of the sections contain studies which would require control groups. Control groups are normally included in biological tests to determine whether any observed effects are attributable to exposure to the test substance. For the purposes of this subdivision of the guidelines, there are three important types of control groups: untreated (negative) controls, vehicle controls, and positive controls. The animals of any of these three control groups would have to be the same age and come from the same source as the comparable animals receiving the test substance. In addition, to prevent any possible bias, animals chosen for the test and control groups would have to be assigned at random. Finally, the control groups recommended by the guidelines would, of course, have to be run concurrently with the groups receiving the test substance.

A vehicle control group would be recommended for nearly all studies. The studies would require animals in the vehicle control group to be treated in a manner identical to the test groups in every respect except for exposure to the test substance. They should receive the same care, diet, and ancillary materials (e.g., vehicle or dust suppressant) and be subjected to the same environmental conditions as the animals exposed to the test substance.

In some cases, the studies would have an untreated (negative) control group, in addition to the vehicle control group. A negative control group is usually necessary to determine whether any effects observed in the vehicle control or test group animals are due to the modifying effects (e.g., synergism, antagonism, or independent toxicity) of the ancillary materials (vehicles) used in the study. Or, if no vehicles are used, the negative control operates as a direct comparison with the test groups used in the study. Thus, animals in the negative control group would be treated like the vehicle control group and test group animals, except that they would receive neither the test substance nor any of the ancillary materials (e.g., vehicle or dust suppressant).

A positive control group, when used, serves as an internal quality control: to ascertain whether the test substance produces an effect similar to a related substance of known toxicity; to ascertain whether laboratory staffs are properly making and recording sophisticated visual determinations and properly carrying out other aspects of the test; and to ascertain if a strain or species reacts similarly to another species or strain when exposed to a known standard toxicant. For example, in delayed neurotoxicity testing, triorthocresylphosphate (TOCP) may be used as a principle against which to measure the neurotoxic effect of a substance. The Agency believes that the use of a positive control with each set of test groups would be quite important for three kinds of studies (acute delayed neurotoxicity, subchronic neurotoxicity, and mutagenicity) due to the sophisticated observations and diagnoses involved.

Data on a fourth kind of control group, historical or colony controls, are also sometimes used in evaluating test results. Historical control data usually give the investigator information about the longevity, fecundity, or incidences of spontaneous tumors and other diseases of one species or strain of animal. Comparison to vehicle or negative control data may also indicate whether the test animals are typical of their species and strain. From a scientific viewpoint, however, it is not satisfactory to rely solely on historical control data for accurate control information. Since historical controls are not run concurrently with the testing of the substance under study, the environmental conditions of the study may be different; the animals may have come from a different breeding colony; or the measurements may have been obtained at different times of the year. Any of these (and many other) differences could affect the reliability of any comparisons between historical controls and test groups. Yet historical data is valuable for certain comparative or statistical purposes, and such data should be submitted when pertinent, or upon Agency request.

The Agency realizes the scientific benefits of vehicle and negative controls run concurrently with the test groups. It also recognizes that both of these control groups may not be necessary for each of the tests proposed in the guidelines. Positive control data, too, are useful, primarily for internal quality control. EPA recognizes, however, that some of the substances used as positive controls may expose the testing personnel to undue risks and create disposal or environmental safety problems.

Using concurrent control groups also increases the number of animals and analytical procedures recommended, and therefore increases the cost of each test performed. In the current guidelines, the Agency has accordingly attempted to recommend the various kinds of control groups as prudently as possible. [See § 80-3(b)(5).]

E. Clinical Laboratory Tests.

Clinical laboratory tests of animals are proposed in a number of studies. (See, e.g., §§ 82-1, 83-1.) Most frequently, EPA would recommend some measurements on blood and urine samples, though occasionally other data, including food consumption and body weight, are requested. EPA considers that data developed from clinical tests give a better indication of the onset and development of toxic effects than can be gained from observation of the animals' behavior alone. In addition, effects detected in clinical tests often can support data generated through post-mortem examination of test animals. Accordingly, EPA believes that some clinical testing is necessary.

F. Observation and Handling of Animals.

All of the guidelines would recommend procedures for observation of test animals. The provisions often establish two criteria relating to frequency of observation. First, appropriately-trained personnel would be expected to observe the animals as frequently as needed to detect behavioral or other observable expressions of toxic effects on an animal. In the case of short-term studies, EPA expects that such observations would occur at least twice a day. Daily observation would be adequate in most cases for studies lasting several weeks or longer. In all studies, however, investigators would be expected to observe animals more frequently if necessary to detect signs of toxicity.

Second, personnel would be expected to observe animals as frequently as necessary to prevent loss of the animals by cannibalism, autolysis, misplacement, and similar management problems. The guidelines specify that sick animals should be moved to separate cages, and moribund animals should be sacrificed. These procedures should help to assure that statistically sufficient numbers of test and control animals are used for the purpose of generating the necessary data.

The principles paragraphs also state that all signs of toxicity observed throughout a study would have to be recorded and reported. In response to early comments that this provision alone was too vague to be useful, EPA has added a list of toxic signs which should be recorded, if observed. This list, however, is not exhaustive, and any other signs noted would also be required to be reported.

G. Extent of Necropsy and Histopathology.

Many of the guidelines sections recommend that at least some of the animals in the study be subjected to gross necropsy and histopathology examination. A necropsy involves dissection of an animal and examination of its tissues for grossly visible indications of toxic effects, such as, for example, an enlarged liver. Necropsy often includes removal and weighing of certain organs, as well. A histopathology examination involves microscopic examination of slides containing tissue from different parts of an animal's body. A histopathology examination will detect changes at the cellular level, such as cancer or degeneration of nerve fiber. To perform necropsies or histopathology examinations properly requires considerable training, skill, and time. Consequently, these procedures are costly, and there are only a relatively limited number of people who are qualified to perform them. These procedures, however, are also a very reliable way of identifying and measuring the toxic effects caused by a pesticide. EPA is concerned with the development of principles which will generate all of the useful information which can reasonably be obtained from necropsies and histopathology examinations without overburdening the nation's testing resources with expensive recommendations.

The principles governing histopathology examinations in subchronic and chronic studies are also generally designed to minimize the number of animals which must be examined while maintaining the sensitivity of the studies. These principles are based on the assumption that, especially in short-term studies, most toxic effects follow a clear dose-response pattern; the incidence and severity of the effect increase at higher dose levels. Accordingly, EPA would recommend in subchronic rodent studies that the tissues of all high dose level and control animals be examined microscopically. 5/ The liver, kidney, and lungs would have to be examined in the intermediate and low dosage level groups, but if no compound-related effects are observed in any other tissue in any high level animal, then such other tissues would not need to be examined microscopically in animals from the lower dose levels. If, however, effects are observed at the high dose level, the tissues showing the effects would have to be examined in all animals in the lower dose levels.

5/ Section 83-1, chronic toxicity study, includes principles for a test using four non-rodents per sex at each dose level. All non-rodents from each dose level must be subjected to a histopathology examination. Non-rodents would be treated differently from rodents in this study because far fewer animals are involved, and because this is the only major study to use non-rodent mammals.

Somewhat more extensive histopathology examinations would be recommended by the chronic toxicity studies, § 83-1. Occasionally, histopathologic effects may not be manifested at the highest dose level, particularly when that level causes significant growth depression; these effects would therefore likely appear at the next lower level. In addition, unlike the younger animals used in subchronic studies, old animals examined at the end of lifetime studies often show a great deal of spontaneous disease involving multiple organ systems. Chemically-induced lesions, intermixed with the effects of disease, may be missed during the gross necropsy. Because of these possibilities, EPA thinks a chronic feeding study should include a more extensive histopathology examination than a subchronic study.

There are two other qualifications to the general approaches described above. First, any tissue from any dose level showing evidence of an effect, either from necropsy or clinical test results, would have to be examined at all dose levels. Second, those tissues which are most often affected by toxic pesticides would have to be examined from every dose level. EPA considers that following these standards will generally detect most toxic effects and minimize the number of animals which would be examined.

Necropsy and histopathology in the Oncogenicity Study and Teratogenicity Study, §§ 83-2 and -3, are handled differently from the other chronic and subchronic studies. No histopathology examination would be expected for teratogenicity testing because most birth defects (terata) are grossly visible in animals at necropsy. Necropsy would be recommended on all animals, since the high dose, which should cause toxic effects in the mothers, may mask the appearance of birth defects. In an oncogenicity study, all animals from all dose levels would be subject to necropsy and histopathology examination, because data from a number of tests have shown that a stronger oncogenic response may be elicited from a lower dose level than from the highest dose level. This anomalous dose-response pattern may be attributable to dose-related differences in pharmacokinetics and metabolism of the chemical or to cellular or tissue destruction at the higher dose level (resulting, in many cases, in early mortality) which may prevent the same level of response that would be elicited at the lower levels.

III. DISCUSSION OF INDIVIDUAL TESTS

This part of the Discussion explains the purposes for requiring data from each of the different kinds of tests and the major issues identified in developing individual guidelines sections.

A. Acute Oral, Dermal, and Inhalation Toxicity; §§ 81-1, -2, -3.

Data from acute oral, dermal, and inhalation toxicity studies are used for a number of purposes. EPA considers these data in determining the relative toxicity of various compounds, how a pesticide causes toxic effects, and the proper design of longer-term studies. In addition, a number of specific regulatory decisions are based on acute toxicity data. The decisions deal with determinations as to whether to: issue a rebuttable presumption against registration (RPAR) [40 CFR § 162.11(a)]; classify a pesticide for general use or restrict it for use only by certified applicators [40 CFR § 162.11(c)]; require special packaging (as proposed in 40 CFR § 162.16); and require that the pesticide product labeling contain certain hazard warnings (40 CFR § 162.10). The kind of hazard warning depends on the toxicity category to which a pesticide belongs. The categories are ranges of toxicity determined at either end by an LD50 or LC50.

The oral, dermal, and inhalation toxicity guidelines sections contain principles which provide that if no mortality is produced by administration of a specified dose level, no further testing is required. (See "Limit test" paragraphs.) In the acute oral and inhalation toxicity guidelines, these dose levels correspond to the lower limit of Toxicity Category IV. No matter how much higher the LD50 or LC50, the substance will be placed in Category IV, and no other regulatory action will be taken on the basis of the product's acute toxicity. In the acute dermal toxicity guidelines section, however, the upper dose level is 2 g/kg, rather than the lower limit for Toxicity Category IV, 20 g/kg. Agency staffs believe that it would be physically impossible to administer a larger dose to rabbits. (It is noted that the Agency is currently in the process of revising its pesticide product labeling regulations to reflect the physical impossibility of dosing at greater than 2 g/kg.) Moreover, Agency staffs are persuaded that no human would be likely to absorb as much as 20 g/kg (approximately 2 1/2 lbs. for the average adult American) of any substance by the dermal route, and that 2 g/kg is a more reasonable approximation of the largest dose which a human could possibly absorb through the skin.

B. Irritation and Sensitization Studies; §§ 81-4, -5, -6.

Data from three studies, primary eye irritation, primary dermal irritation, and dermal sensitization, are used principally to support label precautionary statements. Eye and dermal irritation data, just as acute oral, dermal, and inhalation toxicity data, are used to place a product within a toxicity category. Section 162.10 of the registration regulations requires a product's label to bear particular hazard warnings depending on the products toxicity category. Positive results in a dermal sensitization study, too, will result in requirements for label warnings.

C. Neurotoxicity Evaluations; §§ 81-7 and 82-5.

A neurotoxic pesticide is one which causes damage to the nervous system. Neurotoxicity can occur in many different ways, and, therefore, most guidelines sections require an applicant to look for signs indicating that a pesticide may affect the nervous system.

These guidelines also include two sections which focus exclusively on neurotoxicity. The first, § 81-7, is a special type of test designed to screen for only one of the many different kinds of neurotoxicity, "delayed neurotoxicity." Delayed neurotoxicity is a syndrome typically caused by certain organophosphate chemicals in which damage to nervous system causes unsteady reflexes and eventually can result in paralysis. These signs first appear several days to a few weeks following exposure, and hence the effect is "delayed." EPA's experience indicates that every compound which produces delayed neurotoxicity in any test system will also cause delayed neurotoxicity when given in a very large dose to hens under an acute regimen. Thus, the acute delayed neurotoxicity study would be used to identify these compounds, and if positive results are observed in the study, further testing would be required.

The subchronic guideline section 82-5, was designed primarily for further testing of substances which have been shown to produce organophosphorus-like delayed neuropathy. The test animal is the hen.

Alternate procedures may be required if a substance other than an organophosphorus material is to be tested for delayed neuropathy, or if a substance is to be tested for a neuropathy other than the organophosphorus-type delayed neuropathy. In such situations usually the test animal will not be the hen. Instead, the species in which the neuropathy had been demonstrated might be used. Testing of both sexes might be required.

Such an altered test in which a mammalian species is used might be combined with another subchronic study, provided the purposes of both studies were satisfied.

When a no-observed-effect level for a neuropathy is to be determined and an altered or different procedure is to be used, prior consultation with EPA scientists is advised.

D. Subchronic Studies; §§ 82-1, -2, -3, and -4.

Subchronic studies involve regular repeated exposure to a pesticide, and data from at least one of these studies would be required to support the registration of virtually every product. Data from these studies serve as a bridge between information developed in acute and chronic tests. Two important purposes of subchronic testing are to identify target organs and to establish appropriate dose levels for lifetime studies. In addition, the subchronic studies are often used to evaluate the relative toxicity of a substance using different routes of exposure. Finally, the clinical testing which would be required in subchronic studies is considerably more extensive than that which would be required in chronic studies. These additional data are useful in understanding the development of toxic effects.

This subdivision also contains two different sections describing subchronic dermal toxicity studies. The Repeated Dose Dermal Toxicity: 21-Day

Study, § 82-2, is considerably less extensive than the Subchronic Dermal Toxicity: 90-Day Study, § 82-3. Data from the 21-day study is essential to support the registration of all products whose use is likely to result in repeated human skin contact. This study would be used primarily to assess subchronic local dermal toxicity, that is, effects on the skin at the site of application. Some data on systemic toxicity are also produced by this study; these data would be compared with any available data from a subchronic oral toxicity study to determine whether the pesticide is potentially more toxic by the dermal route.

Data from the 90-day dermal study is essential when human dermal exposure is purposeful, and either the dermal route of exposure appears to be more toxic than the oral route or no subchronic oral data are necessary. If these conditions exist, the Agency considers it necessary to recommend an extensive subchronic dermal study of systemic toxicity. Thus, this section would extend the duration of dosing to 90 days, increase the clinical testing recommendations, and expand the scope of the necropsy and histopathology examinations beyond the standards proposed in the 21-day study. The scope of the 90-day dermal study, then, would be comparable to that of the rodent subchronic oral toxicity study.

E. Chronic Toxicity Studies and Oncogenicity Studies; §§ 83-1, -2, -5.

This subdivision includes three sections which describe tests for detecting the adverse effects resulting from long-term exposure to a pesticide. One of the sections, Oncogenicity Studies (§ 83-2), focuses on the detection of malignant and benign tumors and preneoplastic lesions. Another, Chronic Toxicity Studies (§ 83-1), is designed primarily to evaluate other chronic effects. (While the route of administration of the test substance is generally oral, the study is required to support the registration of products whose use resulted in repeated human exposure by any route.) Nonetheless, despite minor differences in design and purpose, both studies are capable of detecting oncogenic and non-oncogenic effects, and both kinds of effects must be reported, regardless of the study in which they appear. Moreover, because the studies are so similar, they usually can be combined into a single test when the rat is the test species for both. Because of this, standards for a combined study to meet the requirements of both sections has been added at § 83-5, Combined Chronic Toxicity/Oncogenicity Studies. Such a combined study provides for the evaluation of exposure to the test substance in rodents covering the majority of the expected life span of the strain (approximately 24 months duration in rats and 18 months in mice). This is consistent with EPA's policy concerning "food use pesticides," and is based on the Agency's experience in reviewing data from chronic feeding studies in rodents and therefore will remain in effect until further notice.

EPA thinks that use of a non-rodent species in chronic toxicity studies, as well as a rodent species, is necessary to provide an adequate evaluation of non-oncogenic chronic effects (see § 83-1). The non-rodent, usually the dog, may metabolize the test substance differently from the rodent, and therefore the two species, together, may reveal a much broader range of toxic effects than if only one species were tested. [See, generally, Goldenthal, E., "Current Views on Safety Evaluation of Drugs," FDA Papers, 13 (May, 1968); Fitzhugh, O.G., 1965, "Appraisal on the safety of chemicals in foods, drugs, and cosmetics - chronic oral toxicity," pp. 36, 38. Assoc. Food & Drug Offic. of the U.S., Topeka, Kansas.]

EPA believes that conducting oncogenic and chronic feeding studies for predetermined long-term periods is valuable for several reasons. EPA thinks that these time periods are long enough to allow tumors and other chronic effects to develop, and yet short enough to assure that a reasonable percentage of the animals will survive to the scheduled termination point. A high level of survival would provide the experimenter with a larger data base on which to perform statistical analysis. In addition, since geriatric diseases often make diagnosis of neoplastic growth difficult, the studies would be designed to produce meaningful histology by terminating the studies before such diseases normally become a significant problem. By using a relatively uniform duration, EPA would also be better able to compare the results of different studies and to compare results with the historical data base. Finally, the principles establish a fairly definite endpoint for chronic studies which would allow experimenters to plan their studies more efficiently.

F. Teratogenicity and Reproduction Studies; §§ 83-3, -4.

Two studies in the chronic/long-term series are designed to evaluate the effects of a pesticide on the process of reproduction. Data from a Teratogenicity Study, § 83-3, indicate whether exposure to a pesticide during pregnancy can cause fetotoxicity or birth defects in offspring. The Reproduction/Fertility Study, § 83-4, involves exposure to a pesticide and is designed to assess more subtle effects on reproduction, such as decreased fertility, premature delivery, and smaller offspring.

Commenters have suggested that, in addition to the Teratogenicity Study in § 83-3, tests on behavioral and central nervous system (CNS) defects may be useful. Evaluation of defects in the CNS would have to be done in litters during the postnatal period. The need for postnatal teratological evaluation arises mainly because the CNS does not mature fully in the human fetus and may still be liable to certain teratogenic influences in late pregnancy. The studies would involve treating pregnant female animals during the latter third of gestation, observing the neonate throughout sexual maturity, and perhaps performing objective tests on these animals, if appropriate. Comments are encouraged on the characteristics of a suitable test species, number of test animals, dose levels, duration of neonate observation, and tests to be performed on these neonates.

The principles for the reproduction study represent a significant departure from traditional testing procedures. Instead of a three-generation study with two litters in each generation, EPA provides a two-generation study with only one litter in each generation. The Agency considers that this methodology is generally more sensitive than the traditional test design. In addition, this methodology is considerably less expensive than the traditional test design because fewer animals are required.

A traditional reproduction study requires two litters per generation because the first litters produced by adolescent mothers often show a great deal of variation. Because of these variations, data based on the first litters are less useful than data from the second set of litters, which are more uniform in size and health. The current guidelines recommend only one litter, but the animals would not be bred until they had fully matured. Consequently, under the current principles, the first litter would be less likely to show the variability of the first litters produced by juvenile mothers in a traditional study.

In addition to reducing the number of litters per generation, this guideline would reduce the number of generations. Part of the reason reproduction studies traditionally have tested three generations is to detect genetic abnormalities. Under the Subdivision F guidelines, however, the potential of a pesticide to cause genetic damage would be assessed in a battery of mutagenicity tests which are collectively more sensitive than than a three-generation reproduction study. (See also part III. G. of this Discussion.) The other reason for extending a reproduction study into the third generation is to detect cumulative effects. The Agency, however, is not aware of any toxic effect which first appeared in the third generation of a reproduction study. While the current guidelines reduce the number of generations, it would extend the period of dosing for each litter-producing animal. Accordingly, EPA believes this test design to be more sensitive for cumulative effects than the traditional three-generation study.

G. Mutagenicity Studies §§ 84-1 and -2.

Data from mutagencity tests are used for several purposes. Data are used directly to determine if a test substance is genotoxic, that is, capable of interacting with or damaging genetic material (DNA) and/or mechanisms, or mutagenic, and hence capable of producing heritable genetic changes. The Agency recognizes that a mutagen causing a heritable defect in humans has not yet been identified, however the design and conduct of an epidemiologic study to verify such a relatively rare event is difficult. Since mutations do occur in humans, the Agency feels that it needs to collect information in experimental test systems in order to make reasonable decisions regarding risks associated with chemicals which may be capable of inducing human genetic alteration. Mutagencity data may also be used as an indication of the oncogenic potential of a pesticide.

After considering the public comments to the mutagenicity testing requirements in the 1978 FIFRA proposed guidelines (40 CFR 163) the Agency has decided to allow more options in satisfying test requirements. The different tests vary in their ability to detect mutagens depending on the chemical structure or the physical properties of the test substance. In addition many of these tests are still being validated and consequently individual laboratories may prefer to perform assays with which they feel more confident. The Gene-Tox Program of the EPA Office of Toxic Substance (OTS) has made recommendations on major mutagenicity tests and protocols are being finalized for the test standards of OTS and for the international testing programs of OECD. Other protocols may be found in the EPA/SRI International Project "In Vitro Mutagenicity Studies of Environmental Chemicals," as well as in the Report of the International Collaborative Program "Evaluations of Short-Term Tests for Carcinogens" (Progress in Mutation Research vol. 1, ed. by F.J. de Serres and J. Ashby, Elsevier/North Holland, New York, 1981)

In view of these ongoing efforts, the mutagenicity testing subdivision of this document describes the criteria for selecting a battery of tests and lists representative tests which may be acceptable for this battery. Additional tests not listed may also be acceptable if sufficient documentation is furnished for the Agency to validate their usefulness.

For each test substances a battery of tests is required to assess potential to affect the qualitative or quantitative integrity of human genetic material. The objectives underlying the selection of a battery of tests for mutagencity assessment are:

1. To detect, with sensitive assay methods, the capacity of a test substance to alter genetic material in cells,
2. To determine the relevance of these changes to mammals; and, when mutagenic potential is demonstrated,
3. To incorporate these findings in the risk assessment for heritable effects, oncongenicity, and possibly, other health endpoints.

The battery must include tests appropriate to address the following three categories of genetic effects:

(1) gene mutations
(2) structural chromosomal aberrations
(3) other mechanisms of mutagenicity (e.g. spindle inhibition, direct DNA damage) as appropriate for the tested chemical.

Specific battery test selection and protocol design should be submitted to the Agency for comment and evaluation. Registrants are encouraged to discuss results of preliminary testing with the Agency.

§ 85-2 Domestic Animal Safety Testing.

Data from Domestic Animal Safety Studies are to be derived from studies using specific domestic animals (e.g., cat, dog, chicken, cow, pig) as test animals for the various kinds of tests required in this subdivision of the guidelines, such as acute oral, subchronic inhalation, etc. Such data would be required on a case-by-case basis when the Agency cannot satisfactorily evaluate how a pesticide will affect certain domestic species by using data otherwise required by the subdivision. EPA may require such data, for example, when a pesticide, such as a tick collar or louse dust, is being used directly on a domestic animal. When the Agency decides additional data are needed, it will also establish appropriate principles for acceptable testing to generate the data.

§ 85-3 Dermal Absorption Studies of Pesticides.

The skin can be the major route of human exposure to pesticides during application, harvesting and home use; however, dermal absorption studies are not considered routinely necessary. Dermal absorption studies may be required on an individual basis for compounds having a serious toxic effect, identified by oral or inhalation studies, for which a significant route of human exposure is dermal and for which the assumption of 100% aborption does not produce an adequate margin of safety.

Registrants should work closely with the Agency in developing and performing dermal absorption studies. The experience of Agency scientists has identified many problems in published dermal absorption studies which make them inappropriate for pesticides. Dermal absorption protocols developed by the FDA should not be used for pesticides since they are designed specifically for the types of formulations controlled by the FDA.

The Agency has available, on request, a protocol for *in vitro* determination of dermal absorption. This protocol is undergoing practical evaluation in the laboratories of several registrants but it is not considered ready for publication. The Agency is also interested in *in vitro* methods but the Agency scientists involved do not consider themselves ready to write a protocol. Practical suggestions based on hands-on experience in *in vitro* methodology are being sought from individual investigators.

SUBDIVISION F -- HAZARD EVALUATION: HUMANS AND DOMESTIC ANIMALS

Series 80: OVERVIEW, DEFINITIONS, AND GENERAL REQUIREMENTS

[NOTE: The sections in this series are essentially the same as those published in the 1978 proposed guidelines for Subpart F. The current sections offer important and useful guidance to those engaged in developing toxicology data to meet pesticide registration requirements.

IMPORTANT: If the recommendations of any section in this Series 80 differ from those of the specific test sections of Subdivision F or the good laboratory practice requirements of 40 CFR Part 160 (soon to be published) then the latter shall be followed.]

§ 80-1 Overview.

This subdivision details the toxicity data recommended to support the registration of pesticide products. Each section specifies the conditions under which the data are required. These data are evaluated to determine potential adverse toxicological effects to humans and domestic animals as a result of use of a pesticide. Having made these evaluations and determinations, the Agency must then determine whether:

(a) The application for registration should be approved [see § 162.7(d) of the FIFRA sec. 3 regulations];

(b) The pesticide gives rise to a rebuttable presumption against registration [see § 162.11(a)];

(c) The pesticide is a candidate for general or restricted use classification [see § 162.11(c)];

(d) The labeling of the product is adequate to protect field workers and applicators and complies with the requirements of FIFRA (see § 162.10); and

(e) The pesticide product is subject to special packaging requirements (see § 162.16).

§ 80-2 Definitions.

(a) Terms used in this subdivision shall have the meanings set forth at § 162.3 of FIFRA sec. 3, at § 60-2 of Subdivision D, and at § 190-2 of 40 CFR Part 160.

(b) In addition, for the purposes of this subdivision:

(1) The term "pharmacological effect" means any chemically-induced physiological change in a test animal.

(2) The term "target organ" means any organ of a test animal showing evidence of an effect of chemical treatment.

(c) Refer to the individual test sections of this subdivision for definitions of additional terms.

§ 80-3 General provisions.

(a) Scope. The standards contained in this section apply to all studies in this subdivision unless another section of this subdivision contains a specific standard on the same subject. In such a case, the specific standard in the other sections should apply to the conduct of that particular study.

(b) Basic principles for testing.

(1) Personnel.

(i) All testing and evaluation must be done under the direction of personnel who have the education, training, and experience to perform the testing and evaluation in accordance with sound scientific experimental procedures. The Agency may require resumes of personnel who have performed, supervised, reviewed, or evaluated the testing.

(ii) To help assure consistency in the development of data, one person should be responsible for each particular phase of a study. This is especially important with respect to the conduct of necropsies, when several persons are assigned separate tasks in a necropsy procedure.

(A) A Board-Certified or Board-Eligible pathologist or a person with equivalent training, with experience in laboratory animal pathology, should have the final and overall responsibility for all necropsy and histopathology procedures, and for the accuracy and reliability of all diagnoses, conclusions, and reporting.

(B) A properly trained pathology assistant, under the direct supervision of the pathologist, may perform gross necropsy.

(C) A histology technician, such as one certified by the American Society of Clinical Pathology (HTASCP) or one having equivalent training and capability, may be responsible for all histologic preparations.

(iii) An appropriately educated, trained, and experienced toxicologist should be ultimately responsible for the execution of all phases of each study.

(2) Test substance.

(i) Sections 81-1 through 85-2 specify whether the data submitted in support of an application for registration shall be derived from tests conducted with the technical grade of the active ingredient, the end-use product, both, or some other substance.

(ii) The technical grade of the active ingredient is often the same substance as the manufacturing-use product. In this case, where the sections require testing of the technical grade of the active ingredient, a sample of the manufacturing-use product shall be tested. Where this is not the case, the tests shall be conducted with the technical grade of the active ingredient which is used to produce the manufacturing-use or end-use pesticide product.

(iii) The lot of the substance tested should be the same throughout the duration of the study, and the research sample shall be stored under conditions that maintain its purity and stability. If the stability of the test substance cannot be maintained for the duration of the study or if, for other reasons, it is not possible to use the same lot throughout the test, subsequent lots of the test substance can be selected that are as nearly identical to the original lot as practical. Chemical assays shall be performed to assure this identity and permit reporting of any deviation in composition.

(iv) The composition of each lot of the test substance shall be determined, including the name and quantities of known contaminants and impurities, as far as is technically feasible. The determination shall include quantities of unknown materials, if any, so that 100 percent of the test sample is accounted for. The test substance shall be within the limits, if any, certified in accordance with § 62-2.

(v) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture shall be determined prior to the start of the study. No mixture of test or control substance with the feed or vehicle shall be maintained or used during a period exceeding the known stability of the test or control substance in the mixture. Alternatively, determinations of the stability of the test or control substance in statistically randomized samples of the diet or vehicle mixture shall be made periodically during the study to ensure that proper mixing, formulation, and storage procedures are being followed and that the appropriate concentration of the test or control substance is contained in the mixture. (See 40 CFR Part 160, Good Laboratory Practices.)

(vi) If the test substance is incorporated into feed or another vehicle, its homogeneity and concentration shall be determined prior to the start of the study and periodically during the study (40 CFR Part 160, Good Laboratory Practices). Statistically randomized samples of the mixture shall be analyzed to ensure that the proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(vii) In addition to or in lieu of data otherwise requested by this subdivision, the Agency may request, after consultation with the applicant, data derived from testing to be conducted with:

(A) An analytically pure grade of an active ingredient;

(B) The technical grade of an active ingredient;

(C) An inert ingredient of a pesticide formulation;

(D) A contaminant or impurity of an active or inert ingredient;

(E) A metabolite (from animals or plants) or degradation product of an active or inert ingredient;

(F) The pesticide end-use product.

(G) Any additional substance which enhances the toxic activity (up to and including synergistic effects) of the product for which registration is sought; or

(H) Any combination of the test substances mentioned in paragraphs (b)(2)(vii)(A) through (G) of this section.

(3) Administration of substance and vehicles.

(i) The manner of administration of the test and control substance should be selected so as to maintain accuracy of the dosage.

(ii) All doses in a study should be administered to the animals by the same route and method.

(iii) Where dosing is daily, dosing treatments should be conducted at approximately the same time each day.

(iv) If a vehicle is used to dissolve or dilute the test substance or positive control substance, it should be chosen to possess the following characteristics to the greatest degree known:

(A) It does not alter the absorption, distribution, metabolism, or retention of the test substance;

(B) It does not alter the chemical properties of the test substance or enhance, reduce, or alter the toxic characteristics of the chemical;

(C) It does not affect the food and water consumption or the nutritional status of the animals;

(D) At the levels used in the study, it does not produce physiological effects; and

(E) It closely resembles the vehicle, if any, to be used under expected conditions of use.

(4) Control groups. Control groups are used to assure that effects observed are associated or attributed to the test chemical exposure. The appropriate control group shall be identical in every respect to the test group except for exposure to the test substance. Within a given study, all control animals shall be from the same source, be of the same age, receive the same care, and be fed from the same batch and lot during the same period as the animals receiving the test substance. To prevent bias, a system to randomly assign animals to test groups and control groups is required. [See also paragraph (b)(5) of this section.]

(i) Untreated (negative) control group. An untreated control group is usually required. This group receives neither the test substance nor any ancillary material (vehicle). Consult individual sections of this subdivision for those tests where an untreated control is require.

(ii) Vehicle control groups. (A) If a vehicle is used to administer the test substance, a concurrent vehicle control group is recommended. Animals in this group receive treatment with the vehicle alone, usually at the highest level the vehicle is used for any test group in the study. Consult individual sections of this subdivision for those tests where a vehicle control is required.

(B) As provided in paragraph (b)(3)(iv) of this section, the vehicle shall be selected on the basis of information establishing that it is non-toxic at the levels used in the study, has no independent physiological effects, and does not alter the chemistry or toxicity of the test substance. If, however, there are insufficient data on the effects of the vehicle, testing of the vehicle is required.

(iii) Positive control group. Positive control groups generally are not recommended. These groups serve as an internal quality control, to demonstrate whether the test animals are sensitive to or respond in a predictable manner to known toxic agents, and to ascertain if a strain or species reacts similarly to another strain or species when exposed to the same known or standard toxicant. Consult individual sections of this subdivision for those tests where a positive control is recommended.

(iv) Historical (colony) controls. Data on historical controls are required when the Agency desires information on the longevity, spontaneous diseases, and tumor incidences of a species or strain selected for a study, and for certain comparative or statistical purposes. Consult individual sections of this subdivision for those tests where historical control data are required.

(5) Animal care and selection.

(i) Each animal shall be assigned a unique identification number.

(ii) All data submitted in support of an application for registration must be derived from tests conducted in accordance with good laboratory practices. Healthy animals shall be used, and kept in conditions conforming to good husbandry practices. Animals shall be assigned to test groups in such a manner as to minimize bias and assure comparability of pertinent variables. The animals of all test groups shall, as nearly as practicable, be of uniform weight, age, and parity, and should be representative of the species and strain under study. Control animals shall be housed, fed, and handled in a manner identical to that for the test animals, provided, however, that they shall be caged and housed to preclude or minimize airborne or other contamination by the test substance.

(iii) A testing facility shall have a sufficient number of animal rooms or areas to assure separation of species or test systems and isolation of individual projects. In addition, there shall be sufficient rooms to receive, quarantine, and isolate the animals, and to provide for their routine or (when needed) specialized housing. Structural requirements and environmental control of these rooms or areas for animals shall comply with the provisions of the Animal Welfare Act (Pub. L. 94-279) as set forth in 9 CFR § 3. Space requirements for preliminary enclosure shall also be as specified in 9 CFR § 3, except that where specifications regarding housing of certain species of animals are not set forth, the recommendations contained in DHEW Publication No. (NIH) 78-23 entitled "Guide for the Care and Use of Laboratory Animals" shall be used. For long term studies, recommendations of the NAS publication 1138 are appropriate. See also 40 CFR Part 158 and 40 CFR Part 160.

(iv) Feed and water administered to test animals in chronic studies shall be chosen so as to minimize contaminant chemical residues. Also, within a given study, all control animals shall be fed from the same batch and lot, and shall receive water from the same source, during the same time period as animals receiving the test substance. If possible, the feed should be analyzed to assure uniform distribution and adequacy of nutritional components and combinations with other chemical substances (e.g., contaminant pesticides, if present).

(6) Caging of test animals. Animals may be group-caged unless a specific test principle directs otherwise. Minimum space requirements are outlined in DHEW Publication No. (NIH) 78-23. When appropriate, they should be provided with necessary materials for nesting and shelter. Whenever signs of morbidity are observed during the test, such affected animals should be moved to separate cages to avoid cannibalism.

(7) Equipment. All equipment used in conducting the test, including equipment used to prepare and administer the test substance and equipment used to maintain environmental conditions, shall be of appropriate design

and adequate capacity as specified in DHEW Publication No. (NIH) 74-23. Equipment shall be inspected, cleaned, and maintained regularly. The equipment shall be properly calibrated.

(8) Observation and clinical testing.

(i) All observed signs of intoxication and abnormal behavior shall be recorded at the times of observations throughout the study.

(ii) If a particular kind of clinical test is required to be repeated during the test period, the test should be performed on the same animals whenever possible. However, small animals should not be over-used for such tests.

(9) Number of animals for tests. The number of animals prescribed in the principles of the test method for each section will permit adequate evaluation of most toxicological effects. If a toxicological effect occurs with a marginally significant incidence, data from further testing with larger numbers of animals may be required.

(10) Necropsy procedures. If a section of this subdivision recommends necropsy examinations be conducted, the following principles should apply:

(i) Procedures to minimize loss of valuable tissues through autolysis or cannibalism must be employed and should include: undertaking careful clinical examination of animals to detect those approaching death; killing and immediately performing necropsy of moribund animals; and isolating weak animals, to ensure that not more than 10 percent of the animals are lost.

(ii) If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (but not frozen) at temperatures low enough to minimize autolysis.

(iii) Qualified personnel shall be available so that necropsies can be performed immediately or as soon as possible, but generally no later than 16 hours after the time of death.

(iv) Scheduled necropsies shall be performed under the direct supervision of a qualified pathologist. [See paragraph (b)(1)(iii) of this section.] If histopathology examinations of animal tissues and organs are also required, the same pathologist should be responsible for both tasks.

(v) Dead animals and their organs and tissues shall be identified by reference to the animals' identification numbers.

(11) Tissue and microslide preparation.

(i) Fixation.

(A) Tissues and organs destined for microscopic examination should be placed in 10% buffered formalin or a recognized suitable fixative as soon as they are removed from the carcass and have undergone necropsy examination.

(B) Tissues should be fixed no less than 48 hours prior to trimming.

(ii) Trimming. (A) Tissues should be trimmed to a maximum thickness of 0.4 cm for processing.

(B) Parenchymal organs should be trimmed to allow the largest surface area possible for microscopic examination. Hollow organs should be trimmed and blocked to allow a cross section mount to be obtained from mucosa to serosa. All lymph nodes to be examined should be bisected, preferably through the hilus.

(C) Tissue trimming should be performed by a pathologist or by a pathology assistant under the direct supervision of the pathologist. [See paragraph (b)(1)(iii) of this section.]

(iii) Microslide preparation.

(A) Microsections should be routinely 3-5 micrometers thick, and in no case should a microsection thickness exceed 10 micrometers. All tissues should be stained with hematoxylin and eosin; however, the use of special stains appropriate to the individual tissues or lesions is encouraged.

(B) Tissue preparation, block cutting, and slide preparation should be performed by an HTASCP certified technician or a person having equivalent training and capability. [See paragraph (b)(1)(iii) of this section.]

(iv) Identification. Preserved tissues and organs, tissue blocks, and microscopic slides should be identified by reference to the animals' identification numbers.

(12) Quality assurance. The testing laboratory shall develop and maintain a system to assure and document adequate performance of its staff and equipment. This requirement can be met by a Quality Assurance Unit as described in 40 CFR Part 160 of these guidelines, or by some other equivalent system of quality control, such as those described in a recent comprehensive monograph, "Quality Assurance Practices for Health Laboratories" (Inhorn, 1978). Other references for quality assurance are cited in paragraph (d) of this section.

(c) Medical or clinical evidence. The Agency may require further data if medical or clinical evidence or if retrospective epidemiological evidence suggests that a product or any of its ingredients produces a toxicological or pharmacological effect not already reflected in the tests required in this subdivision.

(d) References. The following are a few examples of publications which are available in the rapidly developing field of quality control (quality assurance):

(1) Bermes, E.W., V. Erviti, and D.T. Forman. 1976. Chapter 2. Statistics, normal values and quality control. In Fundamentals of Clinical Chemistry. Tietz, N., ed. W.B. Saunders: Philadelphia.

(2) Dharan, M. 1977. Total Quality Control in the Clinical Laboratory. C.V. Mosby: St. Louis.

(3) Feigenbaum, A.V. 1961. Total Quality Control Engineering and Management. McGraw-Hill: New York.

(4) Galen, R.S., and S.R. Gambino. 1975. Beyond Normality (The Predictive Value and Efficiency of Medical Diagnosis). John Wiley and Sons: New York.

(5) Inhorn, S.L., ed. 1978. Quality Assurance Practices for Health Laboratories. American Public Health Association: Washington, D.C. 20036.

(6) Reed, A.H., and R.J. Henry. 1974. Chapter 12. Accuracy, precision and control charts. In Clinical Chemistry: Principles and Technics. 2nd Ed. Henry, R.J., ed. Harper and Row: New York.

§ 80-4 Reporting of data.

Each test report submitted under this subdivision shall satisfy the reporting requirements of this section, unless a specific section elsewhere in this subdivision directs otherwise.

(a) General requirements.

(1) Identification. Each test shall identify:

(i) The laboratory where the test was performed, by name and address; and

(ii) Each party primarily responsible for any written or other matter contained in the report, and the portions of the report for which he is responsible.

(2) Verification. Each test report shall be:

(i) Signed by each of the senior scientific personnel, including the laboratory director, responsible for performing and supervising the testing, and preparing, reviewing, and approving the test report; and

(ii) Certified by the applicant or an authorized agent of the applicant as a complete and unaltered copy of the report provided by the testing laboratory, whether independent or owned, operated, or controlled by the applicant.

(b) Format and content. The test report shall include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. A test report should contain at least three parts: a summary and evaluation of the test results; a description of the test procedures; and the data and information required by each applicable section of this subdivision. Particular information, data, or analysis may be required more than once in the test report, and it should be reported or referenced each time that it is required. Units of measurement must be in the metric system, but the English system may also be used when appropriate. In no instance should the systems be mixed (e.g., mg/sq. in.) nor should both systems be used alternately within a test report.

(1) Summary and evaluation of test results. This section of the test report shall contain a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. The summary should highlight any and all positive data or observations, and any deviations from control data which may be indicative of toxic effects. The summary should be presented in sufficient detail to permit independent evaluation of the results.

(2) Description of the test procedure. This section of the test report shall include, but not be limited to, the following information. If an applicant believes the reporting requirements are inapplicable, he should submit an explanatory statement to this effect.

(i) Deviation from standards. The report should indicate all ways in which the test procedure fails to meet applicable standards for acceptable testing contained in this subdivision, and should state the reasons for such deviations. (See 40 CFR Part 158.)

(ii) Methodology. Specification of test methods, including a full description of the experimental design and procedures, the length of the study, and the dates on which the study began and ended, shall be stated.

(iii) Substance tested. Identification of the test substance shall be provided, including:

(A) Chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition (including names and quantities of known contaminants and impurities, so far as is technically feasible; the determinations should also include quantities of unknown materials, if any, so that 100 percent of the sample tested is accounted for);

(B) Manufacturer and lot number of the test substance; relevant properties of the substance tested, such as physical state, pH, stability, and purity; and

(C) Identification and composition of any vehicles (e.g., diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance.

(iv) Animal data. Animal data shall include:

(A) Species and strain used, rationale for selection of species (if the species is other than the species preferred or required by sections of this subdivision), and rationale for selection of strain;

(B) Source of supply of the animals;

(C) Description of any pre-test conditioning, including diet and quarantine;

(D) Method of randomization used in assigning animals to test or control groups;

(E) Numbers of animals of each sex in each test or control group; and

(F) Age and condition of animals at beginning of study.

(v) Environmental conditions. A description of the caging conditions shall include: number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of the diet of the test animal.

(vi) Dosing. Dosing information shall include:

(A) All dose levels administered;

(B) Method and frequency of administration (including hour of dosing in relation to photoperiod);

(C) Total volume of material (i.e., test substance plus vehicle) individual dosings;

(D) Duration of treatment;

(E) If the test substance is administered in the feed or by another vehicle, the method of randomization used in selecting samples to assay, the assay method used to determine the stability and homogeneity of the test substance being administered, and the results of this assay;

(vii) Treatment for infectious diseases. A description of the treatment(s) used to prevent or control infectious diseases if such treatment was undertaken during a test or shortly before a test was begun. Such a description shall include, for each individual affected animal:

(A) Its identification number;

(B) The nature and severity of the disease, if present;

(C) The date of first observation and duration of disease, if present;

(D) The nature of the treatment for disease or disease prevention, and the dates of such treatment; and

(E) The outcome of the treatments in relation to the disease and to the test results.

(viii) Observations. Frequency, duration, and method of observation of the animals.

(ix) Availability of raw data, specimens, and samples of the test substances. The location of all raw data, specimens, and samples of the test substances which are retained in accordance with 40 CFR Part 160 and the name and address of the individual who is responsible for the archives.

(x) References. Statistical and any other methods employed for analyzing the raw data, a list of references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results.

(3) Reporting requirements for specific tests. This section of the test report should include all data, information, and analysis required by the "Data reporting and evaluation" paragraphs of the sections in this subdivision.

(c) Statistical procedures.

(1) General. Statistical techniques are required for several toxicological analyses, such as the LD50 calculations for acute oral and acute dermal toxicity studies (§§ 81-1 and 81-2), the LC50 calculations for acute inhalation toxicity study (§ 81-3), and the median particle size analyses used to describe the aerosol clouds in the acute and subchronic inhalation studies (§§ 81-3 and 82-4). Median lethal doses are to be measured within a 95% confidence limit of 20% of their median, when technically feasible. When not feasible, e.g., due to inherently variable responses or to difficulties in administering the test substance, the registration applicant should explain why the limit was exceeded. In addition, appropriate statistical methods shall be used to summarize experimental data, to express trends, and to evaluate the significance of differences in data from individual test groups. The methods used shall reflect the current state of the art. A list of references in paragraph (d) of this section represents some of the techniques currently in use.

(2) Standard deviation and standard error. All data averages or means shall be accompanied by standard deviation, to indicate the amount of variability in the raw data. In addition, the standard errors of the means may also be calculated, since they are useful in comparing means from different test groups; however, notations of statistically significant differences, accompanied by the

confidence level or probability, may be used in place of the standard errors. Other methods of expressing data dispersion may also be used, when appropriate.

(d) References.

(1) The following are a few of many good textbooks in statistics:

(i) Remington, R.D., and M.A. Schork. 1970. Statistics with Applications to the Biological and Health Sciences. Prentice-Hall: New York. [Includes a chapter on non-parametric methods (Chapter 12. Distribution Free and Nonparametric Methods) which are useful for non-normally distributed data].

(ii) Rohlf, F.J., and R.R. Sokal. 1969. Statistical Tables. W.H. Freeman and Company: San Francisco.

(iii) Sokal, R.R., and F.J. Rohlf. 1969. Biometry. W.H. Freeman and Company: San Francisco.

(iv) Von Fraunhofer, J.A., and J.J. Murray. 1976. Statistics in Medical, Dental and Biological Studies. Tri-Med Books Limited: London.

(2) The following are examples of available computer programs which can be used in the statistical processing of data but generally involve a large computer operation. There are also many desk-top minicomputers which supply similar computer programs for statistical analyses.

(i) Dixon, W.J., ed. 1970. Biomedical Computer Programs (BMD). 2nd Ed. University of California Press: Los Angeles.

(ii) Nie, N.H., C.H. Hull, J.G. Jenkins, K. Steinbrenner, and D.H. Bent. 1975. Statistical Package for the Social Sciences (SPSS). 2nd Ed. McGraw-Hill: New York.

§ 80-5 Combined testing.

(a) Policy. In order to encourage efficient use of test animals and laboratory resources, the data required by this subdivision may be derived from test methodologies which satisfy the principles for acceptable testing contained in two or more different sections of this subdivision.

(b) Procedures. (1) Principles of Test Methods. Where combined testing is conducted, the standards for acceptable testing contained in this subdivision for each of the data requirements should be satisfied or, in accordance with Part 158, Subpart B, an applicant should establish that the purposes of the standards would be satisfied by the combined testing protocol.

(2) Consultation. Prior to initiating a combined test methodology, an applicant is encouraged to consult with the Agency to determine whether the proposed combined test methodology would be acceptable.

(c) Combined testing instruction in test sections. Several sections of this subdivision detailing specific test requirements provide specific instruction on combined testing procedures. See especially § 83-5, a section devoted entirely to the combined chronic toxicity - oncogenicity testing.

Series 81: ACUTE TOXICITY AND IRRITATION STUDIES

[NOTE: The sections of this series are prepared in conformity with the guidelines developed by the Organization of Economic Cooperation and Development.]

§ 81-1 Acute oral toxicity study.

(a) When required. Data on the single-dose oral LD50 are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and each end-use product, unless the substance to be tested under paragraph (e) of this section is a gas or highly volatile substance that cannot be administered orally. See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who, as or general rule, is responsible for submission of the required data.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from a single exposure by the oral route. The purpose of the acute oral study is to determine the median lethal dose (LD 50), its statistical limits and slope using a single exposure and a 14-day post-exposure observation period. Data from an acute study serves as a basis for classification, labeling, and packaging. It is also an initial step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance.

(c) Definitions. (1) "Acute oral toxicity" is the adverse effects occurring from the oral administration of a single dose of a substance.

(2) "Dosage" is a general term comprising the dose, its frequency and the duration of dosing.

(3) "Dose" is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(4) "Dose-effect" is the relationship between the dose and the magnitude of a defined biological effect either in an individual or in a population sample.

(5) "Dose-response" is the relationship between the dose and the proportion of a population sample showing a defined effect.

(6) "LD50 (median lethal dose), oral" is a statistically derived single dose of a substance that can be expected to cause death in 50 percent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(d) Principle of the test method. The test substance is administered orally by gavage in graduated doses to several groups of experimental animals, one dose being used per group. Subsequent observations of effects and deaths are made. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied as indicated by the nature of the toxic effects observed. This principle is directed primarily to studies in rodent species but may be adapted for studies in nonrodents.

(e) Substance to be tested. (1) The manufacturing-use product and, if different, the technical grade of each active ingredient shall be tested to support the registration of a manufacturing-use product.

(2) The end-use product shall be tested to support the registration of an end-use product.

(3) The end-use product, as diluted for use in accordance with labeling directions, shall be tested to support the registration of each end-use product intended for domestic application.

(4) If the toxicity of the use dilution or of the end-use product can be established from tests performed on other use dilutions or on other end-use products for which registration is sought, the use dilution need not be separately tested.

(f) Limit test. If a test at one dose level of at least 5000 mg/kg body weight, using the procedures described for the study, produces no compound-related mortality, then a full study using a minimum of three dose levels might not be necessary.

(g) Test procedures. (1) Animal selection. (i) Species and strain. Although several mammalian test species may be used, the rat is the preferred rodent species. Commonly used laboratory strains should be employed. If another species is used, the tester should provide justification/reasoning for its selection.

(ii) Age. Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

(iii) Sex. (A) Equal numbers of animals of each sex are required for each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. At least 10 animals (5 female and 5 male) at each dose level should be used.

(2) Control groups. Neither a concurrent untreated nor vehicle control group is recommended except when the toxicity of the vehicle is unknown.

(3) Dosing. (i) Dose levels and dose selection. At least three dose levels should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose response curve and, where possible, permit an acceptable determination of the LD50.

(ii) Vehicle. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution in oil (e.g., corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

(iii) Volume. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not exceed 1 ml/100 g body weight, except in the cases of aqueous solutions where 2 ml/100 g may be used. Variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

(4) Observation period. The observation period should be for at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time to death are important, especially if there is a tendency for deaths to be delayed.

(5) Exposure. (i) The test substance should be administered in a single dose by gavage, using a stomach tube or suitable intubation cannula.

(ii) Animals should be fasted prior to substance administration. For the rat, food should be withheld overnight; for other rodents with higher metabolic rates a shorter period of fasting is appropriate.

(iii) After the substance has been administered, food may be withheld for a further 3-4 hours.

(iv) If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. Where a dose is administered in fractions over a period, it may be necessary to provide the animals with food and water, depending on the length of the period.

(6) Observation of animals.

(i) A careful clinical examination should be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals.

(iii) Cageside observations should include, but not be limited to, changes in:

(A) The skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory system;

(D) Circulatory system;

(E) Autonomic and central nervous system;

(F) Somatomotor activity; and

(G) Behavior pattern.

(H) Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

(iv) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter and at death. Changes in weight should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(vi) At the end of the test surviving animals are weighed and sacrificed.

(7) Gross pathology. Consideration should be given to performing a gross necropsy of all animals where indicated by the nature of the toxic effects observed. All gross pathological changes should be recorded.

(h) Data and reporting. (1) Treatment of results. Data shall be summarized in tabular form, showing, for each test group:

(i) The number of animals at the start of the test;

(ii) Time of death of individual animals at different dose levels;

(iii) Number of animals displaying other signs of toxicity;

(iv) Description of toxic effects; and

(v) Necropsy findings.

(2) Evaluation of results. The LD50 value shall always be considered in conjunction with the observed toxic effects and any necropsy findings. The LD50 value is a relatively coarse measurement useful only as a reference value for classification, labeling purposes, and packaging, for expressing the possible lethal potential of the test substance by the ingestion route. Reference should always be made to the experimental animal species in which the LD50 value was obtained. An evaluation should include the relationship, if any, between the animals exposed to the test substance and the incidence and severity of all abnormalities, including:

(i) Behavioral abnormalities;

(ii) Clinical abnormalities;

(iii) Gross lesions;

(iv) Body weight changes;

(v) Effects on mortality; and

(vi) Any other toxicological effects.

(3) Test report. In addition to the information recommended by § 80-4, the test report summary shall include the following information:

(i) Tabulation of response data by sex and dose level (i.e., number of animals dying; number of animals showing signs of toxicity; number of animals exposed);

(ii) Description of toxic effects;

(iii) Time of death after dosing;

(iv) LD50 value for each sex determined at 14 days (with the method of determination specified);

(v) Ninety-five percent confidence interval for the LD50;

(vi) Dose-mortality curve and slope (where permitted, by the method of determination);

(vii) Body weights when taken;

(viii) Necropsy findings

(ix) Pathology findings, when performed.

§ 81-2 Acute dermal toxicity study.

(a) When required.

(1) Routine testing. Data on the single-dose dermal LD50 are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and each end-use product, unless the substance which would be tested under paragraph (e) of this section is corrosive or a gas or highly volatile substance that cannot be administered dermally.

(2) Use dilution testing. Data from tests performed with the use dilutions of a product may be required if the use dilution is intended for non-domestic application as a mist or spray. Applicants should consult with the Agency to determine the principles for such testing, if required.

(3) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is useful where exposure by the dermal route is likely. The purpose of the acute dermal study is to determine the median lethal dose (LD50), its statistical limits and slope using a single exposure over a 24-hour period and a 14-day post-exposure observation period. Data from an acute dermal toxicity study serve as a basis for classification, labeling, and packaging. It is also an initial step in establishing a dosage regimen in subchronic and other studies and may provide information on dermal absorption and the mode of toxic action of a substance by this route.

(C) Definitions.

(1) "Acute dermal toxicity" is the adverse effect occurring during or following a 24-hours dermal exposure to a single dose of a test substance.

(2) "Dosage" is a general term comprising the dose, its frequency and the duration of dosing.

(3) "Dose" is the amount of test substance applied. Does is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(4) "Dose-effect" is the relationship between the dose and the magnitude of a defined biological effect either in an individual or in a population sample.

(5) "Dose-response" is the relationship between the dose and the proportion of a population sample showing a defined effect.

(6) "LD50 (median lethal dose) dermal" is a statistically derived single dose of a substance that can be expected to cause death in 50 percent of treated animals when applied to the skin. The LD50 value is expressed in terms of weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(d) Principle of the test method. The test substance is applied to the skin in graduated doses to several groups of experimental animals, one dose being used per group. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied as indicated by the nature of the toxic effects observed.

(e) Substance to be tested.

(1) The manufacturing-use product and, if different, the technical grade of each active ingredient shall be tested to support the registration of a manufacturing-use product.

(2) The end-use product shall be tested to support the registration of an end-use product.

(3) If the toxicity of the end-use product can be established from tests performed on other end-use products, the end-use product for which registration is sought need not be separately tested.

(f) Limit test. If a test at a dose of at least 2000 mg/kg body weight, using the procedures described for this study, produces no compound-related mortality, then a full study using a minimum of three dose levels might not be necessary.

(g) Test procedures. (1) Animal selection. (i) Species and strain. The rat, rabbit or guinea pig may be used. The albino rabbit is preferred because of its size, ease of handling, skin permeability and extensive data base. Commonly used laboratory strains should be employed. If a species other than the three indicated above is used, the tester should provide justification/reasoning for its selection.

(ii) Age. Adult animals should be used. The following weight ranges are suggested to provide animals of a size which facilitates the conduct of the test: rats, 200 to 300 g; rabbits, 2.0 to 3.0 kg; guinea pigs, 350 to 450 g.

(iii) Sex. (A) Equal numbers of animals of each sex with healthy intact skin are recommended for each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. At least 10 animals (5 females and 5 males) at each dose level should be used.

(2) Control groups. Neither a concurrent untreated nor vehicle control group is recommended except when the toxicity of the vehicle is unknown.

(3) Dosing. (i) Dose levels and dose selection. At least three dose levels should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and, where possible, permit an acceptable determination of the LD50.

(ii) Vehicle. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution in oil (e.g., corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

(4) Exposure duration. The duration of exposure should be approximately 24 hours.

(5) Observation period. The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time of death are important, especially if there is a tendency for deaths to be delayed.

(6) Preparation of animal skin. (i) Approximately 24 hours before the test, fur should be removed from the dorsal and ventral area of the trunk of the test animals by clipping or shaving. Care must be taken to avoid abrading the skin which could alter its permeability.

(ii) Not less than 10 percent of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering.

(iii) When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account.

(7) Application of test substance. (i) The test substance should be applied uniformly over an area which is approximately 10 percent of the total body surface area. With highly toxic substances the surface area covered may be less, but as much of the area should be covered with as

thin and uniform a film as possible. In the case where less than 10% of the surface area is covered an approximation of the exposed areas should be determined.

(ii) Test substance should be held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not a recommended method.

(iii) At the end of the exposure period, residual test substance should be removed, where practicable using water or an appropriate solvent.

(8) Observation of animals. (i) A careful clinical examination should be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals.

(iii) Cageside observations should include, but not be limited to, changes in:

(A) Skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory system;

(D) Circulatory system;

(E) Autonomic and central nervous system;

(F) Somatomotor activity; and

(G) Behavior pattern.

(H) Particular attention should be directed to observations of tremors, convulsions, salivation, lethargy, sleep and coma.

(iv) Individual weights of animals should be determined shortly before the test substance is applied, weekly thereafter, and at death. Changes in weight should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(vi) At the end of the test, surviving animals are weighed and sacrificed.

(9) Gross pathology. Consideration should be given to performing a gross necropsy of all animals where indicated by the nature of the toxic effects observed. All gross pathological changes should be recorded.

(h) Data and reporting.

(1) Treatment of results. Data shall be summarized in tabular form, showing, for each test group:

(i) The number of animals at the start of the test;

(ii) Time of death of individual animals at different dose levels;

(iii) Number of animals displaying other signs of toxicity;

(iv) Description of toxic effects; and

(v) Necropsy findings.

(2) Evaluation of results. The dermal LD50 value shall always be considered in conjuction with the observed toxic effects and the necropsy findings. The LD50 value is a relatively coarse measurement, useful as a reference value for classification and labeling purposes, packaging and expressing the possible lethal potential of the test substance following dermal exposure. Reference should always be made to the experimental animal species in which the LD50 value was obtained. An evaluation should include the relationship, if any, between the animals exposed to the the test substance and the incidence and severity of all abnormalities, including:

(i) Behavioral abnormalities;

(ii) Clinical abnormalities;

(iii) Gross lesions;

(iv) Body weight changes;

(v) Effects on mortality; and

(vi) Any other toxicological effects.

(3) Test report. In addition to the information required by § 80-4, the test report summary shall include the following information:

(i) Tabulation of response data by sex and dose level (i.e., number of animals dying, number of animals showing signs of toxicity, number of animals exposed);

(ii) Description of toxic effects;

(iii) Time of death after dosing;

(iv) LD50 value for each sex determined at 14 days (with the method of determination specified);

(v) Ninety-five percent confidence interval for the LD50;

(vi) Dose-mortality curve and slope (where permitted by the method of determination);

(vii) Body weights when taken; and

(viii) Pathology findings, when performed.

(ix) The approximate amount of test material applied per unit of skin exposed (calculated in mg per square cm of skin).

§ 81-3 Acute inhalation toxicity study.

(a) When required.

(1) A determination of the acute inhalation toxicity is required by 40 CFR Part 158 to support the registration of a manufacturing-use product, if:

(i) The product is a gas;

(ii) The product is a solid or a liquid which may produce a significant vapor hazard based on its toxicity and expected use; or

(iii) The product contains particles of inhalable size for man (that is, it contains particles of aerodynamic diameters of 15 micrometers or less).

(2) A determination of the acute inhalation toxicity is required to support the registration of an end-use product, if;

(i) The end-use product (as registered or under conditions of use) is a gas;

(ii) The product is a solid or a liquid which may produce a significant vapor hazard based on its toxicity and expected use; or

(iii) The product under conditions of use will produce inhalable liquid or solid particles (that is, particles of aerodynamic diameter of 15 micrometers or less).

(iv) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of an inhalable material, such as a gas, volatile substance or aerosol/particulate, determination of acute inhalation toxicity is an initial step. It provides information on health hazards likely to arise from short term exposure by the inhalation route. The purpose of the acute inhalation study is to determine the median lethal concentration (LC50), its statistical limits and slope using a single exposure, usually of 4 hours, and a 14-day post-exposure observation period. Data from an acute study serves as a basis for classification and labeling. It is also an initial step in establishing a dosage regimen in subchronic and other studies and may provide additional information on the mode of toxic action of a substance.

(c) Definitions.

(1) "Acute inhalation toxicity" is the total adverse effects caused by a substance following a single uninterrupted exposure by inhalation over a short period of time to a substance capable of being inhaled.

(2) "Aerodynamic diameter" applies to the behavioral size of particles of aerosols. It is in diameter of a sphere of unit density which behaves aerodynamically as the particle of the test substance. It is used to compare particles of different sizes, shapes and densities and to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to "optical," "measured" or "geometric" diameters which are representation of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

(3) "Geometric mean diameter" or "median diameter" is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter and its geometric standard deviation are used to statistically describe the particle size distribution of any aerosol based on the weight and size of the particles.

(4) "Inhalable diameter" refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the deep lung (the aveoli). For man, the inhalable diameter is considered here as 15 micrometers or less.

(5) "LC50" (median lethal concentration) is a statistically derived concentration of a substance that can be expected to cause death during

exposure or within a fixed time after exposure in 50 percent of animals exposed for a specified time. The LC50 value is expressed as weight or volume of test substance per volume of air (e.g., mg/l or ppm).

(d) Principle of the test method. Several groups of experimental animals are to be exposed for 4 hours to the test substance in graduated concentrations, one concentration being used per group. Where a vehicle is required, it ideally should not substantially alter the chemical or toxicological properties of the test substance. Subsequent observations of effects and deaths are made. Animals which die during the tests are necropsied and at the conclusion of the test surviving animals are sacrificed and necropsied as necessary.

(e) Substance to be tested. (1) The manufacturing-use product and, if different, the technical grade of each active ingredient, shall be tested to support the registration of a manufacturing-use product.

(2) The end-use product shall be tested to support the registration of an end-use product.

(3) The chemical composition and physical state of the substance being tested should, if possible, be the same as that which is encountered during the use of the product. Aerosol particles may have to be reduced to sizes which are inhalable for the animal being tested considering the entire respiratory system of the animal.

(f) Limit test. If a test at an exposure of 5 mg/l (actual concentration of respirable substances) for 4 hours or, where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, produces no compound-related mortality, then a full study using three dose levels might not be necessary.

(g) Test procedures. (1) Animal selection. (i) Species and strain. Although several mammalian test species may be used, the preferred species is the rat. Commonly used laboratory strains should be used. If another mammalian species is employed, the tester should provide justification/reasoning for its selection.

(ii) Age. Young adult animals should be used. The weight variation of animals or between groups of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

(iii) Sex. (A) Equal numbers of animals of each sex are recommended for each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. At least 10 animals (5 female and 5 male) at each dose level should be used (except if the limit test applies).

(2) Control groups. Neither a concurrent untreated nor vehicle control group is required except when the toxicity of the vehicle is unknown.

(3) Dose levels and dose selection. (i) At least three exposure concentrations should be used and spaced appropriately (between the 10% and 90% mortality range) to produce test groups with a range of toxic effects and mortality to produce a concentration mortality curve and permit an acceptable determination of an LC50. One dose level with 0% and/or 100% mortalities adjacent to the 10% to 90% dose level may be used to calculate the LC50.

(ii) Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the test substance in the atmosphere. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance.

(iii) In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations.

(4) Exposure duration. The duration of exposure should be at least four hours allowing appropriate additional time for chamber equilibrium.

(5) Observation period. The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time of death are important, especially if there is a tendency for deaths to be delayed.

(6) Inhalation exposure. (i) The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 10 air changes per hour, ensure an adequate oxygen content of at least 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimize crowding of the test animals and maximize their exposure to the test substance. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Maintenance of a slight negative pressure inside the chamber will prevent leakage of the test substance into surrounding area.

(ii) The temperature at which the test is performed should be maintained at 22°C (± 2°) for the rat. The relative humidity should be maintained between 40 to 60 percent unless the nature of the test substance or generating procedure (such as using water as a vehicle) precludes this.

(iii) Alternatively, oro-nasal, or head only exposures may be used if the animals exposed in chambers are excessively coated with test substance and/or the whole body exposures produce high toxicity in the face or low oral or dermal toxicity.

(7) Physical measurements. Measurements and/or monitoring should be made of the following:

(i) The rate of air flow should be recorded at least every 30 minutes. Electronic monitoring of flow is desirable.

(ii) Actual concentrations of the test substance of the atmosphere from the breathing zone of the animals should be determined. Samples should be taken often enough to adequately characterize the atmospheres to which the animals are exposed (at least twice during the exposure, one after initial chamber equilibration and one late in the exposure).

(iii) For exposures to aerosols, aerodynamic particle size analyses should be performed to establish the distribution of the sizes of the particles and the consistancy of the aerosol generating system. Particle size analyses during the animals' test exposures should then be carried out as often as necessary to characterize the aerosols to which the animals are exposed.

(iv) Temperature should be recorded at least every 30 minutes. Electron monitoring of temperature is desirable.

(8) Food and water during exposure period. Food should be withheld during exposure and water should not come in direct contact with the test atmospheres.

(9) Observation of animals. (i) The animals should be observed clinically at least daily and actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals.

(ii) Cageside observations should include, but not be limited to, changes in:

(A) The skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory system;

(D) Circulatory system;

(E) Autonomic and central nervous system;

(F) Somatomotor activity; and

(G) Behavior pattern.

(H) Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

(iii) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death. Changes in weight should be calculated and recorded when survival exceeds one day.

(iv) The time of death should be recorded as precisely as possible.

(v) At the end of the test the surviving animals are weighed and sacrificed.

(10) Gross pathology. Consideration should be given to performing a gross necropsy of all animals where indicated by the nature of the toxic effect observed with particular reference to any changes in the respiratory tract. Where there are significant signs of toxicity indicating the possible involvement of other organs these should be examined and all gross pathological changes recorded.

(11) Histopathology. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 hours or more shall be considered since it may yield useful information.

(h) Data and reporting. (1) Treatment of results. Data should be summarized in tabular form, showing, for each test group:

(i) The number of animals at the start of the test;

(ii) Time of death of individual animals at different exposure levels;

(iii) Number of animals displaying other signs of toxicity;

(iv) Description of toxic effects; and

(v) Necropsy findings.

(2) Evaluation of results. The LC50 value shall always be considered in conjunction with the observed toxic effects and the necropsy findings. The LC50 value is a relatively coarse measurement, useful only as a reference value for classification and labeling purposes, and expressing possible lethal potential of the test substance following inhalation. Reference should always be made to the experimental animal species in which the LC50 value was obtained. An evaluation should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including:

(i) Behavioral abnormalities;

(ii) Clinical abnormalities;

(iii) Gross lesions;

(iv) Body weight changes;

(v) Mortality; and

(vi) Other toxicological effects.

(3) Test report. In addition to the information required by § 80-4, the test report summary shall include the following information:

(i) Test conditions. (A) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, methods of conditioning air, and the method of housing the animals in a test chamber when this apparatus is used.

(B) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size shall be described.

(ii) Exposure data. These shall be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and should include:

(A) Airflow rates through the inhalation equipment;

(B) Temperature of air;

(C) Nominal concentration--total amount of test substance fed into the inhalation equipment divided by volume of air (no standard deviation);

(D) Measured total concentrations (particulate and/or gaseous phases) in test breathing zone; and

(E) Particle size distribution (e.g., median aerodynamic diameter of particles with geometric standard deviation) including estimates of the percents of inhalable and non-inhalable portions for the test animals.

(iii) Animal data. (A) Tabulation of response data by sex and exposure level (i.e., number of animals dying, number of animals showing signs of toxicity, number of animals exposed, species and strain used);

(B) Description of toxic effects;

(C) Time of death during or following exposure;

(D) LC50 for each sex determined at 14 days (with method of calculations specified):

(E) Ninety-five percent confidence interval for the LC50;

(F) Dose-mortality curve and slope (where permitted by the method of determination);

(G) Body weights, when taken; and

(H) Pathology findings, when performed.

§ 81-4 Primary eye irritation.

(a) When required. Data on primary eye irritation are required by 40 CFR Part 158 § support the registration of each manufacturing-use product and each end-use product. See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on eyes of mammals is an important initial step. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the eyes and associated mucous membranes to the test substance.

(c) Definitions.

(1) "Eye corrosion" is the production of irreversible tissue damage in the eye following application of a test substance to the anterior surface of the eye.

(2) "Eye irritation" is the production of reversible changes in the eye following the application of a test substance to the anterior surface of the eye.

(d) Standard of the test method.

(1) The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information. The degree of irritation/corrosion is evaluated and scored at specific intervals and is fully described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 21 days.

(2) Strongly acidic or alkaline substances, for example with a demonstrated pH of 2 or less or 11.5 or greater, need not be tested owing to their predictable corrosive properties.

(3) Materials which have demonstrated definite corrosion or severe irritation in a dermal study need not be further tested for eye irritation. It may be presumed that such substances should produce similarly severe effects in the eyes.

(e) Substance to be tested. (1) Test substance. (i) The manufacturing-use product shall be tested to support the registration of a manufacturing-use product.

(ii) The end-use product shall be tested to support the registration of an end-use product.

(2) Condition of test substance.

(i) If the test substance is a liquid, it should be placed in the eye undiluted, in accordance with paragraph (f)(4) of this section.

(ii) If the test substance is a solid or granular product, it should be ground into a fine dust or powder. The test substance should not be moistened before it is placed in the eye in accordance with paragraph (f)(4) of this section.

(3) Corrosive pesticides. Data which demonstrate that the test substance specified by paragraph (e)(1) of this section has a pH of 1-2 or 11.5-14 may be submitted in lieu of data from a primary eye irritation study conducted in accordance with paragraph (f) of this section. For all regulatory purposes, the Agency will assume that such a substance is corrosive.

(f) Test procedures. (1) Animal selection. (i) Species and strain. A variety of experimental animals have been used, but it is recommended that testing should be performed using healthy adult albino rabbits. Commonly used laboratory strains should be used. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

(ii) Number of animals. At least six animals should be used, unless justification/reasoning for using fewer animals is provided.

(2) Dose level. (i) For testing liquids, a dose of 0.1 ml is used. In testing solids, pastes, and particulate substances, the amount used should have a volume of 0.1 ml, or a weight of not more than 100 mg (the weight must always be recorded). If the test material is solid or granular, it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them, e.g., by tapping the measuring container. To test a substance contained in a pressurized aerosol container the eye should be held open and the test substance administered in a single burst of about one second from a distance of 10 cm directly in front of the eye. The dose may be estimated by weighing the container before and after use. Care should be taken not to damage the eye. Pump sprays should not be used but instead the liquid should be expelled and 0.1 ml collected and instilled into the eye as described for liquids.

(3) Examination of eyes prior to test. (i) Both eyes of each experimental animal provisionally selected for testing should be examined within 24 hours before testing starts by the same procedure to be used during the test examination. Animals showing eye irritation, ocular defects or pre-existing corneal injury should not be used.

(4) Application of the test substance. (i) The test substance should be placed in the conjunctival sac of one eye of each animal after

gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second in order to prevent loss of the material. The other eye, which remains untreated, serves as a control. If it is thought that the substance could cause extreme pain, a local anesthetic may be used prior to instillation of the test substance. The type and concentration of the local anesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anesthetized.

(ii) The eyes of the test animals should not be washed out for 24 hours following instillation of the test substance. At 24 hours, a washout may be used if considered appropriate.

(5) Observation period. (i) The duration of the observation period should not be fixed rigidly, but it should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed. It normally need not exceed 21 days after instillation.

(6) Clinical examination and scoring. (i) The eyes should be examined at 1, 24, 48, and 72 hours. If there is no evidence of irritation at 72 hours, the study may be ended. Extended observation may be necessary if there is persistent corneal involvement or other ocular irritation in order to determine the progress of the lesions and their reversibility or irreversibility. In addition to the observations of the cornea, iris and conjunctivae, any other lesions which are noted should be recorded and reported. The grades of ocular reaction using Table 1 should be recorded at each examination.

TABLE 1: GRADES FOR OCULAR LESIONS

CORNEA

Opacity: degree of density (area most dense taken for reading).

- No ulceration or opacity	0
- Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible	1*
- Easily discernible translucent area, details of iris slightly obscured	2*
- Nacrous area, no details or iris visible, size of pupil barely discernible	3*
- Opaque cornea, iris not discernible through the opacity	4*

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*Starred figures indicate positive effect.

IRIS

- Normal	0
- Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperemia, or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive)	1*
- No reaction to light, hemorrhage, gross destruction (any or all of these)	2*

CONJUNCTIVAE

Redness (refers to palpebral and bulbar conjunctivae, cornea, and iris)

- Blood vessels normal	0
- Some blood vessels definitely hyperemic (injected)	1
- Diffuse, crimson color, individual vessels not easily discernible	2*
- Diffuse beefy red	3*

Chemosis: lids and/or nictating membranes

- No swelling	0
- Any swelling above normal (includes nictating membranes)	1
- Obvious swelling with partial eversion of lids	2*
- Swelling with lids about half closed	3*
- Swelling with lids more than half closed	4*

Discharge

- No discharge	0
- Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
- Discharge with moistening of the lids and hairs just adjacent to lids	2
- Discharge with moistening of the lids and hairs, and considerable area around the eye	3

(ii) Examination of reactions can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable device. After recording the observations at 24 hours, the eyes of any or all rabbits may be further examined with the aid of fluorescein.

(iii) The grading of ocular responses is subject to various interpretations. To promote harmonization and to assist testing laboratories and those involved in making and interpreting the observations, an illustrated guide in grading eye irritation should be used. (Such an illustrated guide is in use in the United States and can be obtained from the Consumer Product Safety Commission, Washington, D.C. 20207.)

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*Starred figures indicate positive effect.

(g) Data and reporting. (1) Data shall be summarized in tabular form, showing, for each individual animal:

(i) The irritation scores at the designated observation time;

(ii) A description of the degree and nature of irritation;

(iii) The presence of serious lesions; and

(iv) Any effects other than ocular which were observed.

(2) Evaluation of the results. The ocular irritation scores shall be evaluated in conjunction with the nature and reversiblity or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They shall be viewed as reference values and are only meaningful when supported by a full description and evaluation of the observations.

(3) Test report. In addition to the information required by § 80-4, the test report shall include the following information:

(i) Physical nature and, where appropriate, concentration and pH value for the test substance;

(ii) Species and strain used;

(iii) Tabulation of irritant/corrosive response data for each individual animal at each observation time point (e.g., 1, 24, 48, and 72 hours);

(iv) Description of any lesions observed;

(v) Narrative description of the degree and nature of irritation or corrosion observed;

(vi) Description of the method used to score the irritation at 1, 24, 48, and 72 hours (e.g., hand slit-lamp, biomicroscope, fluorescein); and

(vii) Description of any non-ocular effects noted.

§ 81-5 Primary dermal irritation.

(a) When required. Data on primary dermal irritation are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and each end-use product. See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on skin of mammals is an important initial step. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance.

(c) Definitions. (1) "Dermal corrosion" is the production of irreversible tissue damage in the skin following the application of the test substance.

(2) "Dermal irritation" is the production of reversible inflammatory changes in the skin following the application of a test substance.

(d) Principle of the test method. (1) The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 14 days.

(2) When testing solids (which may be pulverized if considered necessary), the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

(3) Strongly acidic or alkaline substances, for example with a demonstrated pH of 2 or less or 11.5 or greater, need not be tested for primary dermal irritation, owing to their predictable corrosive properties.

(4) The testing of materials which have been shown to be highly toxic (LD50 less than 200 mg/kg) by the dermal route is unnecessary.

(e) Substance to be tested. (1) Test substance. (i) The manufacturing-use product shall be tested to support the registration of a manufacturing-use product.

(ii) The end-use product shall be tested to support the registration of an end-use product.

(2) Condition of test substance. (i) If the substance is a liquid, it should be applied undiluted.

(ii) If the test substance is a solid, it should be slightly moistened with water or, where necessary, a suitable vehicle before application.

(3) Corrosive pesticides. Data which demonstrate that the test substance specified by paragraph (e)(1) of this section has a pH of 1-2 or 11.5-14 may be submitted in lieu of data from a primary dermal irritation

study conducted in accordance with paragraph (f) of this section. For all regulatory purposes, the Agency will assume that such a substance is corrosive.

(f) Test procedures. (1) Animal selection. (i) Species and strain. The albino rabbit is recommended as the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

(ii) Number of animals. At least six healthy adult animals should be used, unless justification/reasoning for using fewer animals is provided.

(2) Dose level. (i) A dose of 0.5 ml of liquid or 0.5 g of solid or semi-solid is applied to the test site.

(ii) Separate animals are not recommended for an untreated control group. Adjacent areas of untreated skin of each animal serve as control for the test.

(3) Preparation of animal skin. Approximately 24 hours before the test, fur should be removed from the test area by clipping or shaving the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

(4) Application of the test substance. (i) Exposure duration is for four hours. Longer exposures may be indicated under certain conditions, e.g., expected pattern of human use and exposure. At the end of the exposure period, residual test substance should generally be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

(ii) The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In the case of liquids or some pastes, it may be necessary to apply the test substance to the gauze patch and then apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. However, the use of occlusive dressing may be considered appropriate in some cases. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

(5) Observation period. (i) The duration of the observation period is at least 72 hours, but should not be fixed rigidly. It should be sufficient to evaluate fully the reversibility or irreversiblity of the effects observed. It need not normally exceed 14 days after application.

(6) Clinical examination and scoring. (i) After removal of the patch, animals should be examined for signs of erythema and edema and the responses scored within 30-60 minutes, and then at 24, 48 and 72 hours after patch removal.

(ii) Dermal irritation is scored and recorded according to the grades in Table 2, below. Further observations may be needed, as necessary, to establish reversibility. In addition to the observation of irritation, any lesions and other toxic effects should be fully described.

TABLE 2: EVALUATION OF SKIN REACTION

Erythema and Eschar Formation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Maximum possible - 4	

Edema Formation	Value
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 millimeter)	3
Severe edema (raised more than 1 millimeter and extending beyond area of exposure . . .	4
Maximum possible - 4	

(g) Data and reporting. (1) Data shall be summarized in tabular form, showing, for each individual animal:

(i) The irritation scores for erythema and edema at 30 to 60 minutes, 24, 48, and 72 hours after patch removal;

(ii) Any lesions;

(iii) A description of the degree and nature of irritation, corrosion, or reversiblity; and

(iv) Any other toxic effects observed.

(2) Evaluation of results. The dermal irritation scores shall be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They should be viewed

as reference values which are only meaningful when supported by a full description and evaluation of the observations. The use of an occlusive dressing is a severe test and the results are relevant to very few likely human exposure conditions.

(3) Test report. In addition to the information recommended by § 80-4, the test report should include the following information:

(i) Physical nature and, where appropriate, concentration and pH value for the test substance;

(ii) Species and strain used;

(iii) Tabulation of irritation response data for each individual animal for each observation time period (e.g., 30 to 60 minutes, 24, 48, and 72 hours after patch removal);

(iv) Description of any lesions observed;

(v) Narrative description of the degree and nature of irritation observed; and

(vi) Description of any toxic effects other than dermal irritation.

§ 81-6 Dermal sensitization study.

(a) When required. Data from a dermal sensitization study are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and of each end-use product which will result in repeated human skin contact under conditions of use.

(1) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of a substance, determination of its potential to provoke skin sensitization reactions is important. Information derived from tests for skin sensitization serves to identify the possible hazard to a population repeatedly exposed to the test substance. While the desirability of skin sensitization testing is recognized, there are some real differences of opinion about the best method to use. The test selected should be a reliable screening procedure which should not fail to identify substances with significant allergenic potential, while at the same time avoiding false negative results.

(c) Definitions. (1) "Challenge exposure" is an experimental exposure of a previously treated subject to a test substance following an induction period, to determine whether the subject will react in a hypersensitive manner.

(2) "Induction exposure" is an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

(3) "Induction period" is a period of at least one week following a sensitization exposure during which a hypersensitive state is developed.

(4) "Skin sensitization" ("allergic contact dermatitis") is an immunologically-mediated cutaneous reaction to a substance. In the human, the responses may be characterized by pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these. In other species, the reactions may differ and only erythema and edema may be seen.

(d) Principle of the test method. Following initial exposure(s) to a test substance, the animals are subsequently subjected, after a period of not less than one week, to a challenge exposure with the test substance to establish whether a hypersensitive state has been induced. Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction to that of the initial induction exposure.

(e) Substance to be tested. (1) Test substance. (i) The manufacturing-use product shall be tested to support the registration of a manufacturing-use product.

(ii) The end-use product shall be tested to support the registration of an end-use product.

(2) Conditions of test substance. The test substance should be applied at a concentration in accordance with the test methods. If the test substance causes marked irritation, it should be diluted with physiological saline until a concentration is found which produces only slight irritation. If the test substance is a solid to be injected intradermally, it should be dissolved in a minimum amount of physiological saline or suspended in a suitable agent.

(f) Test procedures. (1) Any of the following seven test methods is considered to be acceptable. It is realized, however, that the methods differ in their probability and degree of reaction to sensitizing substances.

(i) Freund's complete adjuvant test;

(ii) Guinea pig maximization test;

(iii) Split adjuvant technique;

(iv) Buehler test;

(v) Open epicutaneous test;

(vi) Mauer optimization test; and

(vii) Footpad technique in guinea pig.

(2) Removal of hair is by clipping, shaving, or possibly by depilation, depending on the test method used.

(3) Animal selection. (i) Species and strain. The young adult guinea pig is the preferred species. Commonly-used laboratory strains should be employed. If other species are used, the tester should provide justification/reasoning for their selection.

(ii) Number and sex. (A) The number and sex of animals used should depend on the method employed.

(B) The females should be nulliparous and non-pregnant.

(4) Control animals. (i) Periodic use of a positive control substance with an acceptable level of reliability for the test system selected is recommended.

(ii) Animals may act as their own controls or groups of induced animals can be compared to groups which have received only a challenge exposure.

(5) Dose levels. (i) The dose level will depend upon the method selected.

(6) Observation of animals. (i) Skin reactions are to be graded and recorded after the challenge exposures at the time specified by the methodology selected. This is usually 24, 48, and 72 hours. Additional notations should be made as necessary to fully describe unusual responses.

(ii) Regardless of method selected, initial and terminal body weights are to recorded.

(7) Procedures. (i) The procedures to be used are those described by the methodology chosen.

(g) Data and reporting. (1) Data summary. Data should be summarized in tabular form, showing, for each individual animal:

(i) The skin reaction; and

(ii) Results of the induction exposure(s) and the challenge exposure(s) at the times indicated by the method chosen.

(2) Grading information. As a minimum, the erythema and/or edema should be graded and any unusual findings should be recorded.

(3) Evaluation of the results. The evaluation of results will provide information on the proportion of each group that became sensitized and the extent (slight, moderate, severe) of the sensitization reaction in each individual animal.

(4) Test report. In addition to the information required by § 80-4, the test report shall include the following information:

(i) A description of the method used and the commonly accepted name;

(ii) Information on positive control study, including:

(A) Positive control used;

(B) Method used; and

(C) Time conducted.

(iii) The number, species, strain and sex of the test animals;

(iv) Individual weights of the animals at the start of the test and at the conclusion of the test;

(v) A brief description of the grading system; and

(vi) Each reading made on each individual animal.

§ 81-7 Acute Delayed Neurotoxicity of Organophosphorus Substances.

(a) Introduction, Purpose, Scope, Relevance, Application and Limits of Test. (1) When Required. As stated in 40 CFR Part 158, organophosphorous substances should be considered as candidates for delayed neurotoxicity studies using the adult hen as the test animal. This test has certain limitations, e.g., in predicting effects from repeated exposures. These limitations may possibly be minimized by conducting an adjunct test in which the inhibition and aging of neurotoxic esterase of hen neural tissue are measured.

(i) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(2) Purpose. This screening procedure is for detecting delayed neurotoxic potential.

(3) Definition. "Acute delayed neurotoxicity" is a prolonged, delayed-onset locomotor ataxia resulting from single administration of the test substance, repeated once if necessary.

(4) Substance Tested. The technical grade of the active ingredient.

(5) Reference Substances. A substance which is known to produce acute delayed neurotoxicity should be used as a positive control. Examples of such substances are tri-orthocresyl phosphate (TOCP) and leptophos.

(6) Principle of the Test Method. The test substance is administered orally in a single dose to domestic hens (Gallus gallus domesticus) which have been protected from acute cholinergic effects. The animals are observed for at least 21 days for delayed neurotoxicity, with redosing and observation for another 21 days if no effects or equivocal responses are seen. The animals are observed daily for behavioral abnormalities, locomotor ataxia and paralysis. Histopathological examination of selected neural tissues is undertaken on all animals surviving the initial cholinergic phases.

(b) Description of the Test Procedure. (1) Preparations. A preliminary LD50 test using an appropriate number of animals, dosages and dose groups, should be performed in unprotected hens to establish the dose level to be used in this test. Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be acclimatized to the laboratory conditions for at least five days prior to randomization and assignment to treatment and control groups.

(2) Experimental Animals. (i) Selection of Species. The adult domestic laying hen, aged between 8-14 months, is recommended. Standard size breeds and strains should be employed.

(ii) Number. A sufficient number of hens should be utilized so that at least six survive the observation period.

(iii) Controls. Appropriate control groups should be used. These should include a positive control group of a least four hens treated with a known delayed neurotoxicant and a concurrent negative control group of at least six hens treated in a manner identical to the test group, except that administration of the test substance and any protective agents is omitted.

(iv) Housing and Feeding Conditions. Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Appropriate diets should be administered as well as an unlimited supply of drinking water.

(3) Test Conditions. (i) Dose Levels. The selected dose level of the test substance should not be less than the unprotected LD50 dose. Atropine or another protective agent demonstrated to be non-interfering

may be used to prevent death due to acute cholinergic effects. Doses of test substance higher than 5000 mg/kg of body weight need not be tested.

(ii) Route of Administration. Dosing with the test substance should normally be by the oral route using gavage or gelatine capsules.

(4) Procedure. The test or control substance should be administered and observations begun. All hens should be carefully observed at least once daily for a period of at least 21 days and signs of toxicity recorded, including the time of onset, degree and duration. Observations should include, but not be limited to, behavioral abnormality, locomotor ataxia and paralysis. At least twice a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to enhance the observation of minimal responses. If neurotoxic responses are not observed or if equivocal responses are seen, then the dose should be administered again and the animals observed for an additional 21 days. The hens should be weighed weekly. Any moribund hens should be removed and sacrificed.

(5) Pathology. (i) Gross Necropsy. Useful information is not usually provided by the results of gross necropsy.

(ii) Histopathology. All animals should be subjected to microscopic examination. Tissues should be fixed in situ, preferably using perfusion techniques. Sections should include medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical bulb, the midthoracic and the lumbo-sacral regions. Sections of the proximal region of the tibial nerve and its branches should be taken. Sections should be stained with appropriate myelin and axon-specific stains.

(C) Data and Reporting. (1) Treatment of Results. Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions or effects, the types of lesions or effects and the percentage of animals displaying each type of lesion or effect.

(2) Evaluation of Results. The findings of an acute delayed neurotoxicity study shall be evaluated in terms of the incidence and severity of neurotoxic effects and of any other observed effects and histopathological findings in the treated and control groups.

(3) Test Report. The test report shall include the following information:

(i) Toxic response data by group with a description of clinical manifestations of nervous system damage; where a grading system is used the criteria should be defined.

(ii) For each animal, time of death during the study or whether it survived to termination.

(iii) The day of observation of each abnormal sign and its subsequent course.

(iv) Body weight data.

(v) Necropsy findings for each animal, when performed.

(vi) A detailed description of all histopathological findings.

(vii) Statistical treatment of results, where appropriate.

(4) Interpretation of Results. This study provides information on the acute delayed neurotoxic effects of exposure to organophosphorus substances. Extrapolation from the results of the study to man is valid only to a limited degree, although it can provide useful information on the degree of neurotoxic activity of a substance.

(5) Literature.

(1) M.B. Abou-Donia, Ann. Rev. Pharmacol. Toxicol 21, 511-548 (1981).

(2) M.B. Abou-Donia and S.H. Pressing, Toxicol. Appl. Pharmacol. 38, 5995-6008 (1976).

(3) EPA-600/176-025 (edited by R.L. Baron), (National Tech. Info. Services, Springfield, VA., 1976).

(4) British Working Documents, October, 2 (Ministry of Agriculture, Fisheries and Food, London, 1967).

(5) J.B. Cavanaugh, CRC Critical Review of Toxicity 2 (3), 365-417 (1973).

(6) F.R. Johannsen, P.L. Wright, D.E. Gordon, G.L. Levinskas, R.W. Radue and P.R. Graham, Toxicol. Appl. Pharmacol. 41, 291-304 (1977).

(7) M.K. Johnson, Arch. Toxicol. 34, 259-288 (1975).

Series 82: SUBCHRONIC TESTING

[NOTE: The sections of this series are prepared in conformity with the guidelines developed by the Organization of Economic Cooperation and Development. Those guidelines were adapted to fit the toxicology data requirements under FIFRA.]

§ 82-1 Subchronic oral toxicity (rodent and non-rodent): 90-day study.

(a) When required. As required by 40 CFR Part 158 data from subchronic oral dosing studies are needed to support the registration of each manufacturing-use product and end-use product that meet either of the following criteria:

(1) The use for which registration application is made requires a tolerance for the pesticide or an exemption from the requirement to obtain a tolerance, or requires the issuance of a food additive regulation; or

(2) The use of the pesticide product is likely to result in repeated human exposure to the product, its active ingredient(s), metabolite(s), or degradation product(s) through the oral route.

(3) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic oral toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic oral study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It should provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(c) Definitions. (1) "Cumulative toxicity" is the adverse effects of repeated doses occuring as a result of prolonged action on, or increased concentration of the administered substance or its metabolites in, susceptible tissue.

(2) "Dose" is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal

(e.g., mg/kg), or as weight of test substance per unit weight of food or drinking water.

(3) "No-effect level"/"No-toxic-effect level"/"No-adverse-effect level"/"No-observed-effect level" is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg). When administered to animals in food or drinking water the no-observed-effect level is expressed as mg/kg of food or mg/ml of water.

(4) "Subchronic oral toxicity" is the adverse effects occurring as a result of the repeated daily oral dosing of a chemical to experimental animals for part (approximately ten percent) of a life span.

(d) Principle of the test method. The test substance is administered orally in graduated daily doses to several groups of experimental animals, one dose level per group, for a period of at least 90 days. During the period of administration, the animals are observed daily to detect signs of toxicity. Animals which die during the period of administration are necropsied, and at the conclusion of the test, all surviving animals are sacrificed and necropsied, and appropriate histopathological examination is carried out.

(e) Substance to be tested. Testing shall be performed with the technical grade of each active ingredient in the product.

(f) Limit test. If a test at one dose level of at least 1000 mg/kg body weight (expected human exposure may indicate the need for a high dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally-related compounds, then a full study using three dose levels might not be necessary.

(g) Test procedures. (1) Animal selection. (i) Species and strain. A variety of rodent species may be used, although the rat is the preferred species. Commonly used laboratory strains should be employed. The commonly used non-rodent species is the dog, preferably of a defined breed; the beagle is frequently used. If other mammalian species are used, the tester should provide justification/reasoning for their selection.

(ii) Age. (A) General. Young, healthy animals should be employed. At the commencement of the study, the weight variation of animals used should not exceed ± 20 percent of the mean weight.

(B) Rodents. Dosing should begin as soon as possible after weaning, ideally before the rats are 6 and, in any case, not more than 8 weeks old.

(C) Non-rodents. In the case of the dog, dosing should be commenced after acclimatization, preferably at 4-6 months and not later than 9 months of age.

(iii) Sex. (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. (A) Rodents. At least 20 animals (10 females and 10 males) should be used at each dose level.

(B) Non-rodents. Eight animals (4 female and 4 male) should be used at each dose level.

(C) Allowance for sacrifice. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before completion of the study.

(D) Number at termination of study. The number of animals at the termination of the study must be adequate for a meaningful evaluation of toxic effects.

(2) Control groups. A concurrent control group is recommended. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(3) Satellite group (rodent). A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

(4) Dose levels and dose selection. (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as a no observed toxic effect level. Therefore, at least three dose levels and a control should be used. Where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used.

(ii) The highest dose level in rodents should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation; for nonrodents, there should be no fatalities.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in the low and intermediate groups and in the controls should be low, to permit a meaningful evaluation of the results. For non-rodents, there should be no fatalities.

(5) Exposure conditions. The animals are treated with test substance ideally for on a 7-days-per-week basis, for a period of 90 days. However, based primarily on practical considerations, dosing in gavage or capsule studies on a 5-days-per-week basis is considered to be acceptable.

(6) Observation period. (i) Duration of observation should be for at least 90 days.

(ii) Animals in the satellite group, if included, scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects.

(7) Administration of the test substance. (i) The test substance may be administered in the diet or in capsules. In addition, for rodents, it may also be administered by gavage or in the drinking water.

(ii) All animals should be dosed by the same method during the entire experimental period.

(iii) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance. It is recommended that, whenever possible, the use of an aqueous solution be considered first, followed by consideration of a solution of oil, and then by possible solution in other vehicles. For non-aqueous vehicles, the toxic characteristics should be known, and if not known, should be determined before the test.

(iv) For substances of low toxicity, it is important to ensure that, when administered in the diet, the quantities of the test substance involved do not interfere with normal nutrition. When the test substance is administered in the diet, either a constant dietary concentration (ppm) or a constant dose level in terms of the animal's body weight, may be used; the alternative used should be specified.

(v) For a substance administered by gavage or capsule, the dose should be given at similar times each day, and adjusted at intervals (weekly or biweekly) to maintain a constant dose level in terms of animal body weight.

(8) Observation of animals.

(i) A careful cageside examination should be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead, and isolation or sacrifice of weak or moribund animals, to ensure that not more than 10% of the animals in any study group are lost from the test due to cannibalism, analysis of tissues, misplacement, and similar management problems.

(iii) Clinical signs of toxicity should be recorded as they are observed including the time of onset, degree and duration.

(iv) Cageside observations should include, but not be limited to, changes in:

(A) Skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory system;

(D) Circulatory system;

(E) Autonomic and central nervous system;

(F) Somatomotor activity; and

(G) Behavior pattern.

(v) Measurements should be made weekly of food and water consumption, depending on the mode of administration of the test substance.

(vi) Animals should be weighed weekly.

(vii) At the end of the 90-day period, all survivors in the non-satellite treatment groups are sacrificed. Any moribund animals should be removed and sacrificed when noticed.

(9) Clinical examinations.

(i) The following examinations should be made on all animals of each sex in each group:

(A) Hematology determinations which are considered to be appropriate to all studies are:

(1) Hematocrit;

(2) Hemoglobin concentration;

(3) Erythrocyte count;

(4) Total and differential leucocyte count; and

(5) A measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Hematology determinations should be investigated for non-rodents at the beginning, then either at monthly intervals or midway through the test period, and, finally, at the end of the test period; for rodents, determinations should be investigated at the end of the test period only.

(C) Clinical biochemistry determinations on blood should be carried out for rodents at the end of the test period, and for non-rodents at the beginning, then either at monthly intervals or midway through the test period, and finally at the end of the test period. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Non-rodents should be fasted for a period (not more than 24 hours) before taking blood samples. Suggested determinations are:

(1) Calcium;

(2) Phosphorus;

(3) Chloride;

(4) Sodium;

(5) Potassium;

(6) Fasting glucose (with period of fasting appropriate to the species);

(7) Serum glutamic-pyruvic transaminase (also known as serum alanine aminotransferase);

(8) Serum glutamic-oxaloacetic transaminase (also known as serum aspartite aminotransferase);

(9) Urea nitrogen;

(10) Albumen;

(11) Blood creatinine;

(12) Total bilirubin; and

(13) Total serum protein measurements.

(14) Other determinations which may be necessary for an adquate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin, and cholinesterase activity.

(15) Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(ii) The following examinations should be made on high dose and control animals of each sex in each group:

(A) Opthalmological examination, using an opthalmoscope or equivalent suitable equipment. This examination should be made prior to the administration of the test substance and at the termination of the study, preferably in all animals, but at least in the high dose and control groups. If changes in the eyes are detected all animals should be examined.

(B) Urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

(10) Gross necropsy.

(i) All animals should be subjected to a full gross necropsy which includes examination of:

(A) The external surface of the body.

(ii) At least the liver, kidneys, and testes should be weighed wet as soon as possible after dissection to avoid drying. In addition, for the non-rodent, the thyroid with parathyroids should be weighed wet.

(11) Tissue preservation.

(i) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(A) All gross lesions;

(B) Brain - including sections of medulla/pons, cerebellar cortex, and cerebral cortex;

(C) Pituitary;

(D) Thyroid parathyroid;

(E) Thymus;

(F) Lungs, trachea;

(G) Heart;

(H) Bone marrow (either femur, sternum or rib at the costochondral junction);

(I) Salivary glands;

(J) Liver;

(K) Spleen;

(L) Kidneys;

(M) Adrenals;

(N) Pancreas;

(O) Testes;

(P) Uterus;

(Q) Aorta;

(R) Esophagus;

(S) Stomach;

(T) Duodenum;

(U) Jejunum;

(V) Ileum;

(W) Caecum;

(X) Colon;

(Y) Rectum;

(Z) Urinary bladder;

(AA) Representative lymph node;

(BB) Peripheral nerve; and

(CC) Gall bladder (if present).

(ii) The following tissues need be preserved only if indicated by signs of toxicity or target organ involvement:

(A) Trachea;

(B) Sternum with bone marrow;

(C) Mammary gland;

(D) Thigh musculature;

(E) Eyes;

(F) Femur - including articular surface;

(G) Spinal cord at three levels - cervical, midthoracic, and lumbar;

(H) Exorbital lachrymal glands (rodent); and

(J) Gall bladder (non-rodent).

(12) Histopathology.

(i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed in paragraph (g)(11) of this section, of all rodents in the control and high dose groups, all non-rodents, and all rodents that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(ii) The following histopathology should be performed. Further histopathological examination may not be recommended on the animals in these groups, but must always be carried out in organs which showed evidence of lesions in the high dose group. These organs should be preserved for future histopathological examination.

(A) Lungs of rodents in the low and intermediate dose groups (special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals);

(B) Liver of all animals in the low and intermediate dose groups; and

(C) Kidneys of all animals in the low and intermediate dose groups.

(iii) For rodents, when a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

(h) Data and reporting.

(1) Treatment of results.

(i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical method shall be selected during the design of the study.

(2) Evaluation of the study results.

(A) The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation should include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including:

(1) Behavioral abnormalities;

(2) Clinical abnormalities;

(3) Gross lesions;

(4) Identified target organs;

(5) Body weight changes;

(6) Effects on mortality; and

(7) Any other general or specific toxic effects.

(8) A properly conducted subchronic test shall provide a satisfactory estimation of a no-effect level.

(B) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(3) Test report. In addition to the information required by § 80-4, the test report summary shall include the following information:

(i) Toxic response and other effects data by sex and dose;

(ii) Species, strain, and/or breed;

(iii) Individual animal data for the following:

(A) Time of death during the study or whether animals survived to termination;

(B) Time of observation of each abnormal sign and its subsequent course;

(C) Food or water consumption data;

(D) Body weight data;

(E) Results of ophthalmological examination;

(F) Hematological tests and all results;

(G) Clinical biochemistry tests and all results;

(H) Necropsy findings;

(I) Detailed description and classification of all histopathological findings; and

(iv) Statistical method applied and treatment of results.

§ 82-2 Repeated dose dermal toxicity: 21 day study.

(a) When required. Data from a subchronic 21-day dermal toxicity study are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and each end-use product whose pesticidal use is likely to result in repeated human skin contact with the product, its active ingredients, or their breakdown products. Data from this study are not required when data from a subchronic 90-day dermal toxicity study (see § 82-3) are required.

(i) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing. It provides information on possible health hazards likely to arise from repeated exposures by the dermal route over a limited period of time.

(c) Definitions.

(1) "Cumulative toxicity" is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of the administered substance or its metabolities in susceptible tissues.

(1) "Dose" in a dermal test is the amount of test substance applied to the skin. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(2) "No-effect level"/"No-toxic-effect level"/"No-adverse-effect level"/"No-observed-effect level" is the maximum dose used in a test which produces no adverse effects. A no-effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg).

(d) Principle of the test method. The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group, for a period of 21 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied and appropriate histopathological examinations carried out.

(e) Substance to be tested. The technical grade of each active ingredient in the product shall be tested. The end-use product shall also be tested when any component of the end-use product is likely to increase dermal absorption of the test substance or potentiate toxic and pharmacologic effects.

(f) Limit test. If a test at one dose level of at least 1000 mg/kg body weight (but expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects, and if toxicity would not be expected based upon data of structurally-related compounds, then a full study using three dose levels might not be necessary.

(g) Test procedures.

(1) Animal selection.

(i) Species and strain. The adult rat, rabbit or guinea pig may be used. Other species may be used but their use would require justification.

(ii) Age. Adult animals should be used. The following weight ranges at the start of the test are suggested in order to provide animals of a size which facilitates the conduct of the test:

(A) Rats, 200 to 300 g.

(B) Rabbits, 2.0 to 3.0 kg.

(C) Guinea pigs, 350 to 450 g.

(iii) Sex. (A) Equal numbers of animals of each sex with healthy skin should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. At least 10 animals (5 females and 5 males) should be used at each dose level.

(2) Control groups. A concurrent control group is recommended. This group should be an untreated or sham treated control group, or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended.

(3) Satellite group. A satellite group of 10 animals (5 animals per sex) may be treated with the high dose level for 21 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 14 days post-treatment.

(4) Dose levels and dose selection. (i) At least three dose levels, with a control and, where appropriate, a vehicle control, should be used.

(ii) The highest dose level should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects.

(v) In the low and intermediate groups and in the controls the incidence of fatalities should be low, to permit a meaningful evaluation of the results.

(vi) If application of the test substance produces severe skin irritation, the concentration may be reduced although this may result in a reduction in, or absence of, other toxic effects at the high dose level. However, if the skin has been badly damaged early in the study it may be necessary to terminate the study and undertake a new study at lower concentrations.

(5) Exposure conditions. The animals are treated with the test substance, ideally for at least 6 hours per day on a 7-days-per-week basis, for a period of 21 days. However, based primarily on practical considerations, application on a 5-days-per-week basis is considered to be acceptable.

(6) Observation period. Duration of observation should be for at least 21 days.

(7) Preparation of animal skin. (i) Shortly before testing, fur is clipped from the dorsal area of the trunk of the test animals. Shaving may be employed but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care must be taken to avoid abrading the skin which could alter its permeability.

(ii) Not less than 10 percent of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering.

(iii) When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account.

(8) Application of the test substance. (i) The test substance should be applied uniformly over an area which is approximately 10 percent of the total body surface area. With highly toxic substances the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

(ii) During the exposure period the test substance is held in contact with the skin with a porous gauze dressing and nonirritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilization is not a recommended method.

(9) Observation of animals. (i) A careful cageside examination should be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals, to ensure that not more than 10% of the animals in any test group are lost from the test due to cannibalism, analysis of tissues, misplacement, and similar management problems.

(iii) Signs of toxicity should be recorded as they are observed including the time of onset, the degree and duration.

(iv) Cageside observations should include, but not be limited to, changes in:

(A) Skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory system;

(D) Circulatory system;

(E) Autonomic and central nervous system;

(F) Somatomotor activity; and

(G) Behavior pattern.

(v) Animals should be weighed weekly. Food consumption data should be collected weekly.

(vi) At the end of the study period all survivors in the non-satellite treatment groups are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(10) Clinical examinations. The following examinations should be made on all animals:

(i) Hematology, including:

(A) Hematocrit;

(B) Hemoglobin concentration;

(C) Erythrocyte count;

(D) Total and differential leucocyte count; and

(E) A measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count, at the end of the test period.

(ii) Clinical biochemistry determinations on blood should be carried out at the end of the test period. Blood parameters of liver and kidney function are appropriate. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are:

(A) Calcium;

(B) Phosphorus;

(C) Chloride;

(D) Sodium;

(E) Potassium;

(F) Fasting glucose (with period of fasting appropriate to the species);

(G) Serum glutamic-pyruvic transaminase (also known as serum alanine aminotransferase);

(H) Serum glutamic-oxaloacetic transaminase (also known as serum aspartite aminotransferase);

(I) Urea nitrogen;

(J) Albumen;

(K) Blood creatinine;

(L) Total bilirubin; and

(M) Total serum protein measurements.

(N) Other determinations which may be necessary for an adequate toxicological evalution include analyses of lipids, hormones, acid/base balance, methemoglobin, cholinesterase activity.

(O) Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(iii) Urinalysis is not required on a routine basis, but only when there is an indication of obtaining useful data based on expected or observed toxicity.

(11) Gross necropsy.

(i) All animals should be subjected to full gross necropsy which includes examination of:

(A) The external surface of the body;

(ii) The liver, kidneys and testes should be weighed wet as soon as possible after dissection to avoid drying.

(12) Tissue preservation. The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(i) Normal and treated skin;

(ii) Liver;

(iii) Kidney; and

(iv) Target organs (i.e., those organs showing gross lesions or changes in size, which are suspected to be related to the treatment of the test substance).

(13) Histopathology. Histological examination should be performed on the preserved organs and tissues of the high dose group and the control group. These examinations should be extended to animals of other dosage groups, if considered necessary to investigate the changes observed in the high dose group. Animals in the satellite group if included should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

(h) Data and reporting. (1) Treatment of results. (i) Data shall be summarized in tabular form, showing for each test group:

(A) The number of animals at the start of the test;

(B) The number of animals showing lesions;

(C) The type of lesions; and

(D) The percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical method shall be selected during the design of the study.

(2) Evaluation of results. The findings of a subchronic dermal toxicity study shall be considered in terms of the observed toxic effects and the necropsy and histopathological findings.

(i) The evaluation should include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including:

(A) Behavioral abnormalities;

(B) Clinical abnormalities;

(C) Gross lesions;

(D) Identified target organs;

(E) Body weight changes;

(F) Effects on mortality; and

(G) Any other general or specific toxic effects.

(ii) A properly conducted 21-day study should provide information on the effects of repeated dermal application of a substance and can indicate the need for further longer term studies.

(iii) It can also provide information on the selection of dose levels for longer term studies.

(3) Test report. In addition to the information required by § 80-4, the test report summary shall include the following information:

(i) Toxic response and other effects data by sex and dose.

(ii) Species and strain.

(iii) Individual animal data for the following:

(A) Time of death during the study or whether animals survived to termination;

(B) The time of the observation of each abnormal sign and its subsequent course;

(C) Body weight data;

(D) Hematological tests employed and results;

(E) Clinical biochemistry tests employed and results;

(F) Necropsy findings;

(G) Detailed description of all histopathological findings; and

(iv) Statistical treatment of results, where appropriate.

§ 82-3 Subchronic dermal toxicity: 90-day study.

(a) When required. Data from a subchronic 90-day dermal toxicity study are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and end-use product whose use will involve purposeful application to the human skin or whose pesticidal use will result in comparable human exposure to the product, its active ingredients, or their breakdown products (e.g., swimming pool algaecides, pesticides for impregnating clothing), and which meets either of the following criteria:

(1) Data from a subchronic oral study (see § 82-1) are not required; or

(2) The active ingredient of the product is known or expected to be metabolized differently by the dermal route of exposure than by the oral route, and a metabolite of the active ingredient is the toxic moiety.

(3) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. In the assessment and evaluation of the toxic characteristics of a chemical the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic dermal study has been designed to permit the determination of the toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening).

(c) Definitions. (1) "Cumulative toxicity" is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of the administered substance or its metabolites, in susceptible tissues.

(2) "Dose" in a dermal test is the amount of test substance applied to the skin (applied daily in subchronic tests). Dose is expressed as weight of the test substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(3) "No-effect level"/"No-toxic-effect level"/"No-adverse-effect level"/"No-observed-effect level" is the maximum dose used in a test which produces no adverse effects. A no-effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg).

(4) "Subchronic dermal toxicity" is the adverse effects occurring as a result of the repeated daily dermal application of a chemical to experimental animals for part (approximately 10 percent) of a life span.

(d) Principle of the test method. The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group, for a period of 90 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied, and appropriate histopathological examinations carried out.

(e) Substance to be tested. The technical grade of each active ingredient in the product shall be tested. In addition, the end-use product shall be tested if any component of the end-use product is likely to increase dermal absorption of the test substance and potentiate toxic or pharmacologic effects.

(f) Limit test. If a test at one dose level of at least 1000 mg/kg body weight (expected human exposure may indicate the need for a high dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally-related compounds, then a full study using three dose levels might not be necessary.

(g) Test procedures. (1) Animal selection. (i) Species and strain. The adult rat, rabbit or guinea pig may be used. Other species may be used but their use would require justification.

(ii) Age. Adult animals should be used. The following weight ranges at the start of the test are suggested in order to provide animals of a size which facilitates the conduct of the test:

(A) Rats, 200 to 300 g.

(B) Rabbits, 2.0 to 3.0 kg.

(C) Guinea pigs, 350 to 450 g.

(iii) Sex.

(A) Equal numbers of animals of each sex with healthy skin should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. (A) At least 20 animals (10 females and 10 males) should be used at each dose level.

(B) If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before completion of the study.

(C) The number of animals at the termination of the study should be adequate for a meaningful evaluation of toxic effects.

(2) Control groups. A concurrent control group is recommended. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended.

(3) Satellite group. A satellite group of 20 animals (10 animals per sex), if included, may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence, of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

(4) Dose levels and dose selection. (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as a no-observed-toxic effect level. Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used.

(ii) The highest dose level should result in toxic effect but not produce severe skin irritation or an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects.

(v) In the low and intermediate groups, and in the controls, the incidence of fatalities should be low to permit a meaningful evaluation of the results.

(5) Exposure conditions. The animals are treated with test substance, ideally for at least 6 hours per day on a 7-days-per-week basis, for a

period of 90 days. However, based primarily on practical considerations, application on a 5-days-per-week basis is considered to be acceptable.

(6) Observation period. Duration of observation should be for at least 90 days.

(7) Preparation of animal skin. (i) Shortly before testing, fur is clipped from the dorsal area of the trunk of the test animals. Shaving may be employed, but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care must be taken to avoid abrading the skin, which could alter its permeability.

(ii) Not less than 10 percent of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering.

(iii) When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account.

(8) Application of the test substance. (i) The test substance should be applied uniformly over an area which is approximately 10 percent of the total body surface area. With highly toxic substances the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

(ii) During the exposure period the test substance is held in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not a recommended method.

(9) Observation of animals. (i) A careful cageside examination should be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals, to ensure that not more than 10% of the animals in any test group are lost from the test due to cannibalism, autolysis of tissues, misplacement, and similar management problems.

(iii) Signs of toxicity should be recorded as they are observed, including the time of onset, the degree, and duration.

(iv) Cageside observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behavior pattern.

(v) Animals should be weighed weekly. Food consumption data should be collected weekly.

(vi) At the end of the study period all survivors in the non-satellite treatment groups are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(10) Clinical examinations. (i) The following examinations should be made on all animals of each sex in each group:

(A) Hematology determinations should be investigated at the end of the test period. Test areas which are considered to be appropriate to all studies are:

(1) Hematocrit;

(2) Hemoglobin concentration;

(3) Erythrocyte count;

(4) Total and differential leucocyte count; and

(5) A measure of clotting potential, such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations on blood should be carried at the end of the test period. Test areas which are considered appropriate to all studies are:

(1) Electroyte balance;

(2) Carbohydrate metabolism; and

(3) Liver and kidney function.

(C) The selection of specific tests will be influenced by observations of the mode of action of the substance. Suggested determinations are:

(1) Calcium;

(2) Phosphorus;

(3) Chloride;

(4) Sodium;

(5) Potassium;

(6) Fasting glucose (with period of fasting appropriate to the species);

(7) Serum glutamic-pyruvic transaminase (also known as serum alanine aminotransferase);

(8) Serum glutamic-oxaloacetic transaminase (also known as serum aspartite aminotransferase);

(9) Urea nitrogen;

(10) Albumen;

(11) Blood creatinine;

(12) Total bilirubin; and

(13) Total serum protein measurements.

(14) Other determinations which may be necessary for an adquate toxicological evalution include analyses of lipids, hormones, acid/base balance, methemoglobin, cholinesterase activity.

(15) Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(ii) The following examinations should be made on all animals of each sex in each group:

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment. This examination should be made prior to exposure to the test substance and at the termination of the study, preferably in all animals but at least in the high dose and control groups. If changes in the eyes are detected, all animals should be examined.

(B) Urinalysis, but only when there is an indication of obtaining useful data based on expected or observed toxicity.

(11) *Gross necropsy*. (i) All animals shall be subjected to a full gross necropsy which includes examination of:

(A) The external surface of the body.

(ii) The liver, kidneys, and testes should be weighed wet, as soon as possible after dissection to avoid drying.

(12) *Preservation of tissues*. (i) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(A) Normal and treated skin;

(B) All gross lesions;

(C) Brain - including sections of medulla/pons, cerebellar cortex and cerebral cortex;

(D) Pituitary;

(E) Thyroid/parathyroid;

(F) Thymus;

(G) Lungs;

(H) Heart;

(I) Salivary glands;

(J) Liver;

(K) Spleen;

(L) Kidneys;

(M) Adrenals;

(N) Pancreas;

(O) Testes;

(P) Accessory genital organs, uterus;

(Q) Aorta;

(R) Gall bladder (if present);

(S) Esophagus;

(T) Stomach;

(U) Duodenum;

(V) Jejunum;

(W) Ileum;

(X) Caecum;

(Y) Colon;

(Z) Rectum;

(AA) Urinary bladder;

(BB) Representative lymph node;

(CC) Peripheral nerve; and

(DD) Aorta.

(ii) The following tissues need be preserved only if indicated by signs of toxicity or target organ involvement:

(A) Trachea;

(B) Sternum with bone marrow;

(C) Mammary gland;

(D) Thigh musculature;

(E) Eyes;

(F) Femur - including articular surface;

(G) Spinal cord at three levels - cervical, midthoracic, and lumbar; and

(H) Exorbital lachrymal glands.

(13) Histopathology. (i) The following histopathology should be performed:

(A) Full histopathology on normal and treated skin and on organs and tissues, listed in paragraphs (e)(12)(i) and (ii) of this section, of all animals in the control and high dose groups.

(B) All gross lesions in all animals.

(C) Target organs in all animal groups.

(ii) The following histopathology should be performed:

(A) Lungs of animals (rats) in the low and intermediate dose groups should be subjected to histopathological examination for evidence of infection, since this provides a convenient assessment of the state of health of the animals.

(B) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in other treated groups.

(h) Data and reporting. (1) Treatment of results. (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical method should be selected during the design of the study.

(2) Evaluation of results. The findings of a subchronic dermal toxicity study shall be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings.

(i) The evaluation will include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including:

(A) Behavioral abnormalities;

(B) Clinical abnormalities;

(C) Gross lesions;

(D) Identified target organs;

(E) Body weight changes;

(F) Effect on mortality; and

(G) Any other general or specific toxic effects.

(ii) A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level.

(3) Test report. In addition to the information required by § 80-4, the test report summary shall include the following information:

(i) Toxic response and other effects data by sex and dose;

(ii) Species and strain; and

(iii) Individual animal data for the following:

(A) Time of death during the study or whether animals survived to termination;

(B) Time of observation of each abnormal sign and its subsequent course;

(C) Body weight data;

(D) Hematological tests employed and results, with relevant baseline data, if available;

(E) Clinical biochemistry tests employed and results, with relevant baseline data, if available;

(F) Necropsy findings;

(G) Detailed description of all histopathological findings; and

(iv) Statistical treatment of results, where appropriate.

§ 82-4 Subchronic inhalation toxicity: 90-day study.

(a) When required. Data from a subchronic inhalation study conducted with the substance specified in paragraph (e) are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and formulated product for which an acute inhalation toxicity determination is required under §163.81-3, and whose pesticidal use may result in repeated inhalation exposure.

(i) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. The subchronic inhalation study is designed to determine the non-observed effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the inhalation route over a limited period of time. It should provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels of chronic studies and for establishing safety criteria for human exposure. Hazards of inhaled substances are influenced by the inherent toxicity and by physical factors such as volatility, particle size and exposure.

(c) Definitions. (1) "Aerodynamic diameter" applies to the behavioral size of particles of aerosols. It is the diameter of a sphere of unit density which behaves aerodynamically as the particle of the test substance. It is used to compare particles of different sizes, shapes and densities to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to "optical," "measured," or "geometric diameter" which is a representation of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

(2) "Geometric mean diameter" or "median diameter" is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter and its geometric standard deviation is used to statistically describe the particle size distribution of any aerosol based on the weight and size of the particles.

(3) "Inhalable diameter" refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the deep lung (the aveoli). For humans, inhalable diameter is considered here as 15 micrometers or less.

(4) "No-effect level"/"Non-toxic-effect level"/"No-adverse-effect level"/"No-observed-effect level" is the maximum dose used in a test which produces no observed adverse effects. It is expressed in terms of weight or volume of the substance per unit volume of air (mg/l or ppm).

(5) "Subchronic inhalation toxicity" is the adverse effects which follow repeated daily exposure of experimental animals to a chemical by inhalation for a significant part of an animal's life span (usually less than 10%).

(d) Principle of the test method. Several groups of experimental animals are exposed daily for defined periods (usually 6 hours per day, 5 days per week) to the test substance in graduated concentrations, one concentration being used per group, for a period of 90 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are sacrificed and necropsied and appropriate histopathological examinations and laboratory analyses carried out.

(e) Substance to be tested. The technical grade of the product shall be tested. The chemical composition and physical state of the substance being tested should, if possible, be the same as that which is encountered during the use of the product. Aerosol particles may have to be reduced to sizes which are inhalable for the animal being tested considering the entire respiratory system of the animal.

(f) Test procedures. (1) Animal selection. (i) Species and strain. A variety of rodent species may be used although the rat is the perferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection. Commonly used laboratory strains should be employed.

(ii) Age. Young adult animals should be used. At the commencement of the study the weight variation of animals should not exceed ± 20 percent of the mean weight for each sex.

(iii) Sex. (A) Equal numbers of animals of each sex should be used at each dose level.

(B) Females should be nulliparous and non-pregnant.

(iv) Numbers. (A) At least 20 animals (10 female and 10 male) should be used for each test group.

(B) If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(C) The number of animals at the termination of the study must be adequate for a meaningful evaluation of toxic effects.

(2) Control groups. One concurrent control group is recommended. If no vehicle is used, an air control group should be treated in the same manner as all other test animals except this control group should not be exposed to an atmosphere containing test substance. When a vehicle is used to help generate the exposure atmosphere, the control group (vehicle control) should be exposed to the greatest concentration of the vehicle used in the study. Ideally, the vehicle should not significally affect the toxicological response of the animals exposed to the test substance.

(3) Satellite group. A satellite group of 20 animals (10 animals per sex), if included, may be treated with the high concentration level and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

(4) Dose levels and dose selection. (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as a no-observed-toxic-effect level. Therefore, at least three concentrations with a control should be used.

(ii) The highest concentration should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest concentration should exceed this.

(iv) Ideally, the intermediate dose level should produce minimal observable toxic effects. If more than one intermediate dose level is used, the concentrations should be spaced to produce a gradation of toxic effects.

(v) In the low and intermediate groups and in the controls the incidence of fatalities should be low, in order to permit a meaningful evaluation of the results.

(vi) In the case of potentially explosive test substances care should be taken to avoid generating explosive concentrations.

(5) Exposure durations. Animals should be exposed to the test substance at least 6 hours per day 5 days per week over a 90 day period. Longer or more continuous exposures may be selected, depending on the test substance and its expected use pattern. If shorter or less continuous exposures seem appropriate, applicants should consult with the Agency concerning the exposure times.

(6) Observation period. (i) Duration of observation should be for at least 90 days. (ii) Animals in a satellite group scheduled for follow-up observations should be kept for at least an additional 28 days without treatment to detect recovery from, or persistence of, toxic effects.

(7) Inhalation equipment. (i) The animals should be tested in inhalation equipment designed to substain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of a least 19 per cent and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimize crowding of the test animals and maximize their exposure to the test substance. As a general rule, to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Maintenance of a slight negative pressure inside the chamber will prevent leakage of the test substance into surrounding area.

(ii) The temperature at which the test is performed should be maintained at 22°C (± 2°) for the rat. The relative humidity should be maintained between 40 and 60 percent, unless the nature of the test substance or generating procedure (such as using water as a vehicle) precludes this.

(iii) Alternatively, oro-nasal or head only exposures may be used if animals exposed in chambers are excessively coated with test substance and/or the whole body exposures produce high toxicity in the face or low oral and dermal toxicity.

(8) Physical measurements. Measurements or monitoring should be made of the following:

(i) The rate of air flow should be recorded at least every 60 minutes. Electronic monitoring of air flow is desirable.

(ii) Actual concentrations of the test atmosphere from the breathing zone of the animals should be determined. Samples should be taken often enough to characterize the atmosphere to which the animals are exposed (at least one determination per run).

(iii) In the case of aerosols, particle size analyses of the exposure atmospheres should be carried out as often as necessary to characterize the aerosols to which the animals are exposed (at least once per run).

(iv) Chamber temperature should be recorded at least once every 60 minutes. Electron monitoring of temperature is desirable.

(9) Food and water. Food should be withheld during exposure and water should not come in direct contact with the test atmospheres.

(10) Observation of animals.

(i) The animals should be observed clinically at least daily and actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals.

(ii) Signs of toxicity should be recorded as they are observed, including the time of onset, the degree and duration.

(iii) Cageside observation should include, but not be limited to, changes in the:

(A) Skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory;

(D) Circulatory;

(E) Autonomic and central nervous systems;

(F) Somatomotor activity; and

(G) Behavior pattern.

(v) Animals should be weighed weekly, and food consumption determined weekly (if body weight is affected).

(vi) At the end of the exposure period all survivors in the non-satellite treatment groups are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(11) Clinical examinations. (i) The following examinations should be made on at least 10 animals of each sex in each group:

(A) Hematology determinations at a minimum should be carried out at the end of the test period. Determinations which are considered to be appropriate to all studies are:

(1) Hematocrit;

(2) Hemoglobin concentration;

(3) Erythrocyte count;

(4) Total and differential leucocyte count; and

(5) A measure of clotting potential, such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) At a minimum, blood chemistry determinations should be done at the end of the test period. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are:

(1) Calcium;

(2) Phosphorus;

(3) Chloride;

(4) Sodium;

(5) Potassium;

(6) Fasting glucose (with period of fasting appropriate to the species);

(7) Serum glutamic-pyruvic transminase (also known as serum alanine aspartite aminotransferase);

(8) Urea nitrogen;

(9) Albumen;

(10) Blood creatinine;

(11) Total bilirubin; and

(12) Total serum protein measurements.

(C) Other determinations which may be necessary for an adquate toxicological evaluation included analyses of lipids, hormones, acid/base balance, methemoglobin, and cholinesterase activity.

(D) Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(ii) The following examinations should be made on at least 10 animals of each sex in each group:

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to exposure to the test substance and at the termination of the study, preferably in all animals, but at least in the high dose and control groups.

(B) Urinalysis on rodents are not suggested as routine procedures but should be carried out when there is a need based on expected or observed toxicity. Among the parameters which should be assessed are color, specific gravity or osmolarity, pH, protein, glucose, ketones, formed elements (RBC's, WBC's, epithelial cells, etc.), crystalline and amorphous materials, and blood pigments.

(12) Gross pathology. (i) All animals should be subjected to a full gross necropsy which includes examinations of:

(A) The external surface of the body;

(B) All orifices; and

(C) The cranial, thoracic and abdominal cavities and their contents.

(ii) At least the liver, kidneys, lungs, and testes should be weighed wet, as soon as possible after dissection to avoid drying.

(13) Tissue preservation.

(i) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(A) Nasopharyngeal tissues;

(B) All gross lesions;

(C) Brain - including sections of medulla/pons, cerebella cortex, and cerebral cortex;

(D) Pituitary;

(E) Thyroid/parathyroid;

(F) Thymus;

(G) Trachea;

(H) Lungs, which should be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure);

(I) Heart;

(J) Salivary glands;

(K) Liver;

(L) Spleen;

(M) Kidneys;

(N) Adrenals;

(O) Pancreas;

(P) Testes;

(Q) Uterus;

(R) Aorta;

(S) Gall bladder (if present);

(T) Esophagus;

(U) Stomach;

(V) Duodenum;

(W) Jejunum;

(X) Ileum;

(Y) Caecum;

(Z) Colon;

(AA) Rectum;

(BB) Urinary bladder;

(CC) Representative lymph node;

(DD) Peripheral nerve;

(EE) Sternum with bone marrow; and

(FF) Eyes.

(ii) The following tissues need be preserved only if indicated by signs of toxicity or target organ involvement:

(A) Skin;

(B) Accessory genital organs;

(C) Mammary gland;

(D) Thigh musculature;

(E) Femur - including articular surface;

(F) Spinal cord at three levels - cervical, midthoracic, and lumbar; and

(G) Exorbital lachrymal glands.

(14) Histopathology.

(i) The following histopathology should be performed:

(A) Full histopathology on the respiratory tract and other organs and tissues, listed in paragraph (f)(13) of this section, of all animals in the control and high dose groups;

(B) All gross lesions in all animals; and

(C) Target organs in all animals.

(ii) The following histopathology should be performed: (A) Lungs of animals in the low and intermediate dose groups should also be subjected to histopathological examination, primarily for evidence of infection since this provides a convenient assessment of the state of health of the animals.

(B) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in other treated groups.

(g) Data and reporting. (1) Treatment of results. (i) Data shall be summarized in tabular form, showing, for each test group:

(A) The number of animals at the start of the test;

(B) The number of animals showing lesions;

(C) The types of lesions; and

(D) The percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical method should be selected during the design of the study.

(2) Evaluation of results. The findings of a subchronic inhalation toxicity study shall be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings.

(i) The evaluation should include the relationship between the concentration of the test substance and duration of exposure, and the presence or absence, the incidence and severity, of abnormalities, including:

(A) Behavioral abnormalities;

(B) Clinical abnormalities;

(C) Gross lesions;

(D) Identified target organs;

(E) Body weight changes;

(F) Effects on mortality; and

(G) Any other general or specific toxic effects.

(ii) A properly conducted 90-day subchronic test shall provide a satisfactory estimation of a no-effect level.

(3) Test report. In addition to the information recommended by § 80-4, the test report summary shall include the following information:

(i) Test conditions. (A) Description of exposure apparatus, including:

(1) Design;

(2) Type;

(3) Dimension;

(4) Source of air;

(5) System for generating particulates and aerosols;

(6) Method of conditioning air; and

(7) The method of housing animals in a test chamber when this is used.

(B) The equipment for measuring temperature, humidity and particulate aerosol concentrations and size shall be described.

(ii) Exposure data. These shall be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and shall include:

(A) Airflow rates through the inhalation equipment;

(B) Temperature and humidity of air;

(C) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air);

(D) Actual concentration in test breathing zone; and

(E) Particle size distribution (e.g., median aerodynamic diameter of particles with deviation from the mean).

(iii) Animal data. (A) Toxic response and other effects data by sex and concentration;

(B) Species and strain used; and

(C) Individual animal data for the following:

(1) Time of death during the study or whether animals survived to termination;

(2) Time of observation of each abnormal sign and its subsequent course;

(3) Body weight data;

(4) Hematological tests employed and all results;

(5) Clinical biochemistry tests employed and all results;

(6) Necropsy findings;

(7) Detailed description and all histopathological findings; and

(D) Statistical treatment of results where appropriate.

§ 82-5 Subchronic Delayed Neurotoxicity: 90-Day Study.

(a) When Required. As required by 40 CFR § 158.134, subchronic delayed neurotoxicity studies shall be conducted if the acute delayed neurotoxicity test showed neuropathy or neurotoxicity. See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(1) Definition.

"Subchronic delayed neurotoxicity" is a prolonged, delayed-onset locomotor ataxia resulting from repeated daily administration of the test substance.

(2) Controls.

If a positive control is used, a substance which is known to produce delayed neurotoxicity should be employed. Examples of such substances are tri-orthocresyl phosphate (TOCP) and leptophos.

(3) Principle of the Test Method.

Multiple dose levels of the test substance are administered orally to domestic hens (Gallus gallus domesticus) for 90 days. The animals are observed at least daily for behavioral abnormalities, locomotor ataxia and paralysis. Histopathological examination of selected neural tissues is undertaken at the termination of the test period.

(b) Description of the Test Procedure.

(1) Preparations.

Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be acclimatized to the laboratory conditions for the least five days prior to randomization and assignment to treatment and control groups.

(2) Experimental Animals.

(i) Selection of Species.

The adult domestic laying hen, aged between 8-14 months, is recommended. Standard size breeds and strains should be employed.

(ii) Number.

Ten hens should be used for each treatment and control group.

(iii) Controls.

A concurrent control group should be used. This group should be treated in a manner identical to the treated group, except that administration of the test substance is omitted.

(iv) Housing and Feeding Conditions.

Cages or enclosures which are large enough to permit mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Appropriate diets should be administered as well as an unlimited supply of drinking water.

(3) Test Conditions.

(i) Dose Levels.

At least three dose levels should be used in addition to the control group(s). The highest dose level should result in toxic effects, preferably delayed neurotoxicity, but not produce an incidence of fatalities which would prevent a meaningful evaluation. The lowest dose level should not produce any evidences of toxicity.

(ii) Route of Administration.

Oral dosing each day for at least five days per week should be carried out, preferably by gavage or administration of gelatin capsules.

(4) Procedures.

The test or control substance should be administered and observations begun. All hens should be carefully observed at least once daily throughout the test period. Signs of toxicity should be recorded, including the time of onset, degree and duration. Observations should include, but not be limited to, behavioral abnormality, locomotor ataxia and paralysis. At least once a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to enhance the observation of minimal responses. The hens should be weighed weekly. Any moribund hens should be removed and sacrificed.

(5) Pathology.

(i) Gross necropsy.

Useful information is not usually provided by the results of gross necropsy.

(ii) Histopathology.

Tissues from all animals should be fixed in situ, using whole animal perfusion techniques. Sections should include medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical bulb, the mid-thoracic and lumbo-sacral regions. Sections of the proximal region of the tibial nerve and its branches and of the sciatic nerve should be taken. Unused portions of the whole brain should be retained for possible future histopathology. Sections should be stained with appropriate myelin and axon-specific stains. Microscopic examination should be carried out on all hens in the control and high-dose groups. Microscopic examination should also be carried out on hens in the low and intermediate dose groups when there is evidence of effects in the high-dose group.

(C) Data and Reporting.

(1) Treatment of Results.

Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions or effects, the types of lesions or effects, the percentage of animals displaying each type of lesion or effect. All observed results should be evaluated by statistical method if appropriate. Any generally accepted statistical method may be used.

(2) Evaluation of Results.

The findings of a subchronic delayed neurotoxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the incidence and severity of observed neurotoxic effects and any other observed effects and histopathological findings in the treated and control groups. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level based on lack of clinical signs and histopathological changes.

(3) Test Report.

The test report shall also include the following information:

(i) Doses administered (mg/kg);

(ii) Toxic response data by group with a description of clinical manifestations of nervous system damage where a grading system is used the criteria should be defined;

(iii) Time of death during the study or whether animals survived to termination;

(iv) The day of observation of each abnormal sign and its subsequent course;

(v) Body weight data;

(vi) Necropsy findings for each animal, when performed;

(vii) A detailed description of all histopathological findings; and

(viii) Statistical treatment of results.

(4) Interpretation of Results.

This study provides information on the neurotoxic effects of repeated exposure to organophosphorous substances. Extrapolation from the results of the study to man is valid only to a limited degree, although it can provide useful information on the degree of neurotoxic activity of a substance, no-effect levels and permissible human exposure.

(5) Literature.

(1) M.B. Abou-Donia, Ann. Rev. Pharmacol. Toxicol. 21, 511-548 (1981)

(2) M.B. Abou-Donia and S.H. Pressig, Toxicol. Appl. Pharmocol. 38, 5995-6008 (1976).

(3) EPA-600/1-76-025 (edited by R.L. Baron) (National Techn. Info. Service, Springfield, VA, 1976).

(4) British Working Documents, October, 2, (Ministry of Agriculture, Fisheries and Food, London, 1967).

(5) J.G. Cavanaugh, CRC Critical Reviews of Toxicology 2 (3), 465-417 (1973).

(6) F.R. Johannsen, P.L. Wright, D.E. Gordon, G.L. Levinskas, R.W. Radue and P.R. Graham, Toxicol. Appl. Pharmacol. 41, 291-304 (1977).

Series 83: CHRONIC AND LONG-TERM STUDIES

[NOTE: The sections of this series are prepared in conformity with the guidelines developed by the Organization of Economic Cooperation and Development. Those guidelines were adapted to fit the toxicology data requirements under FIFRA.]

§ 83-1 Chronic toxicity studies.

(a) When required. As required by 40 CFR § 158.135, chronic feeding studies shall be conducted support the registration of each manufacturing-use product and end-use product that meets either of the following criteria:

(1) Use of the pesticide product is likely to result in repeated human exposure to the product, its active ingredient(s) metabolites, or degradation products over a significant portion of the human life-span (for example, products intended for use in and around residences, swimming pools, and enclosed working spaces or their immediate vicinity); or

(2) The use requires a tolerance for the pesticide or an exemption from the requirment to obtain a tolerance, or requires issuance of a food additive regulation.

(3) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Principle of Test Method. The objective of a chronic toxicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. Under the conditions of this test, effects which require a long latent period or are cumulative should become manifest. The application of these standards should generate data on which to identify the majority of chronic effects and to determine dose-response relationships. Ideally, the design and conduct should allow for the detection of general toxicity including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

(b) Combined studies. For studies designed to meet the requirements of both the chronic toxicity studies and the oncogenicity study, refer to § 83-5.

(d) Substance to be tested. Testing shall be performed with the technical grade of each active ingredient in the product.

(e) Test procedures. (1) Animal selection. (i) Species and strain. Testing should be performed with two mammalian species, a rodent and a non-rodent. The rat is the preferred rodent species and the dog is the preferred non-rodent species. Commonly-used laboratory strains should be employed. If other mammalian species are used, the tester should provide justification/reasoning for their selection.

(ii) Age. (A) Dosing of rats should begin as soon as possible after weaning, ideally before the rats are six, but in any case not more than eight weeks old.

(B) Dosing of dogs should begin between four and six months of age and in any case no later than nine months of age.

(iii) Sex. (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. (A) For rodents, at least 40 animals (20 females and 20 males), and for non-rodents (dogs), at least eight animals (four females and four males) should be used at each dose level.

(B) If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(C) The number of animals in any group should not fall below 50 percent at 15 months in mice and 18 months in rats. At the termination of the experiment at 18 month in mice and 24 months in rats the survival in any group should not fall below 25 percent.

(2) Control groups. (i) A concurrent control group should be utilized. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended.

(ii) In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group is treated in the same manner as all other test animals except that this control group should not be exposed to the test substance or any vehicle.

(3) Dose levels and dose selection. (i) In chronic toxicity tests, it is desirable to have a dose response relationship as well as a no-observed-toxic-effect level. Therefore, at least three dose levels should be used in addition to the concurrent control group.

(ii) The highest dose level in rodents should elicit some signs of toxicity without causing excessive lethality; for non-rodents, there should be no fatalities.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest dose level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results. For non-rodents, there should be no fatalities.

(4) Observation period. The duration of the exposure period in rodents for chemicals intended for a non-food use should usually be a least 12 months while the duration of exposure in rodents for a food-use chemical should be at least 24 months. The duration of exposure to non-rodents should be 12 months.

(5) Administration of the test substance. The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

(i) Oral studies. Provided it can be shown that the test substance is absorbed from the gastrointestinal tract, the oral route of administration is preferred.

(A) The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 12 months.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(C) For a diet mixture, the highest concentration should not exceed five percent with the exception of nutrients.

(D) Ideally daily dosing on a 7-day per week basis should be used because dosing in gavage or capsule studies on a 5-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a 5-day per week basis is considered to be acceptable.

(ii) Dermal studies. (A) The animals are treated by topical application with the test substance, ideally for at least six hours per day.

(B) The test substance should be applied uniformly over a shaved area which is approximately ten percent of the total body surface area. With highly toxic substances the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

(C) Between applications, the test substance is held in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

(iii) Inhalation studies. (A) The animals are exposed by inhalation.

(B) The temperature at which the test is performed should be maintained at 22C (± 2). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols) this may not be practicable. Food and water should be withheld during exposure.

(C) A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(6) Observation of animals. (i) A careful clinical examination should be made at least once each week.

(iv) Daily cageside observations should include, but not be limited to, changes in:

(A) Skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory;

(D) Circulatory;

(E) Autonomic and central nervous system;

(F) Somatomotor activity; and

(G) Behavior pattern.

(iii) Clinical signs of toxicity including suspected tumors and mortality should be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals, to ensure that not more than 10% of the animals in any test group are lost from the test due to cannibalism, analysis of tissues, misplacement, and similar management problems.

(v) Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

(vi) Measurements of food or water consumption should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

(vii) At the end of the study period all survivors are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(7) Physical measurements. For inhalation studies, measurements or monitoring should be made of the following:

(i) The rate of air flow should be monitored continuously and recorded at intervals of at least once every 30 minutes.

(ii) During the exposure period the actual concentrations of the test substance should be held as constant as practicable.

(iii) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution.

(iv) Temperature and humidity should be monitored continuously but should be recorded at intervals of at least once every 30 minutes.

(8) Clinical pathology. (i) The following examinations should be made on at least ten rats of each sex per dose level and on all non-rodents:

(A) Hematology examinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at approximately six month intervals during the conduct of the study and at study termination, on blood samples collected from all non-rodents, and from ten rats/sex of all groups. If possible, these collections should be from the same rats at each interval. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count should be performed on samples from those animals in the highest dosage group and the controls. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls. If hematological effects were noted in the subchronic test, hematological testing should be performed at 3, 6, 12, 18, and 24 months for a two-year study and at 3, 6, and 12 months for a one-year study.

(B) Certain clinical biochemistry determinations on blood should be carried out at least three times: at the beginning, middle, and at the end of the test period. Blood samples should be drawn for clinical chemistry measurements from all non-rodents and at least ten rodents/sex of all groups, if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are:

(1) Calcium;

(2) Phosphorus;

(3) Chloride;

(4) Sodium;

(5) Potassium;

(6) Fasting glucose (with period of fasting appropriate to the species);

(7) Serum glutamic-pyruvic transaminase (also known as serum alanine aminotransferase);

(8) Serum glutamic-oxaloacetic transaminase (also known as serum aspartite aminotransferase);

(9) Blood urea nitrogen;

(10) Albumen;

(11) Blood creatinine;

(12) Creatine phosphokinase;

(13) Total cholesterol

(14) Total bilirubin; and

(15) Total serum protein measurements.

(16) Other determinations which may be necessary for an adquate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin, and cholinesterase activity.

(17) Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(ii) The following should be performed on at least ten rats of each sex per dose level and all non-rodents:

(A) Urine samples should be collected at the same intervals as in the hematological examination described in paragraph (e)(8)(i)(A) of this section. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents:

(1) Appearance;

(2) Volume;

(3) Specific gravity;

(4) Protein;

(5) Glucose;

(6) Ketones;

(7) Bilirubin;

(8) Occult blood (semi-quantitatively); and

(9) Microscopy of sediment (semi-quantitatively).

(B) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study, preferably in all animals, but at least in the high dose and control groups. If changes in the eyes are detected, all animals should be examined.

(10) Gross necropsy. (i) A complete gross examination should be done in all animals, including those which died during the experiment or were killed in moribund condition.

(ii) At least the liver, kidneys, brain and testes should be weighed wet as soon as possible after dissection to avoid drying. For these organs, at least ten rodents per sex per group and all non-rodents should be weighed.

(iii) If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

(11) Tissue preservation. The following organs and tissues, or representative samples there of, should be preserved in a suitable medium for possible future histopathological examination:

(A) Skin;

(B) All gross lesions and tumors;

(C) Brain - including sections of medulla/pons, cerebellar cortex, and cerebral cortex;

(D) Pituitary;

(E) Thyroid/parathyroid;

(F) Thymus;

(G) Lungs/trachea;

(H) Heart;

(I) Sternum and/or femur with bone marrow;

(J) Salivary glands;

(K) Liver;

(L) Spleen;

(M) Kidneys;

(N) Adrenals;

(O) Pancreas;

(P) Testes;

(Q) Accessory genital organs, uterus;

(R) Female mammary gland;

(S) Musculature;

(T) Esophagus;

(U) Stomach;

(V) Duodenum;

(W) Jejunum;

(X) Ileum;

(Y) Caecum;

(Z) Colon;

(AA) Rectum;

(BB) Urinary bladder;

(CC) Lymph nodes;

(DD) Peripheral nerve;

(EE) Spinal cord at three levels -- cervical, midthoracic, and lumbar; and

(FF) Eyes;

(GG) Gall bladder; and

(HH) Aorta.

(ii) In special studies such as inhalation studies, the entire respiratory tract should be studied, including nose, pharynx, and larynx.

(iii) Although inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues, the inflation of the lungs in inhalation studies is essential for appropriate histopathological examination.

(12) Histopathology. (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed in paragraph (e)(11) of this section, of:

(1) All non-rodents;

(2) All rodents in the control and high dose groups; and

(3) All rodents that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(ii) The following histopathology should be performed:

(A) Lungs in all animals; special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals;

(B) Liver in all animals; and

(C) Kidneys in all animals.

(iii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iv) In case the result of the experiment gives evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic respone, the next lower dose level should be examined as described above.

(v) An attempt should be made to correlate gross observations with microscopic findings.

(c) Data and reporting. (1) Treatment of results. (i) Data shall be summarized in tabular form, showing for each test group:

(A) The number of animals at the start of the test;

(B) The number of animals showing lesions;

(C) The types of lesions; and

(D) The percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical method should be selected during the design of the study.

(2) Evaluation of study results. (A) The findings of a chronic toxicity study shall be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation should include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including:

(1) Behavioral abnormalities;

(2) Clinical abnormalities;

(3) Gross lesions;

(4) Identified target organs;

(5) Body weight changes;

(6) Effects on mortality; and

(7) Any other general or specific or chronic toxic effects.

(B) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailabilty of the test substance should be considered.

(3) Test report. In addition to information required by § 80-4, the test report summary shall include the following information:

(i) Toxic response and other effects data by sex and dose;

(ii) Species, strain, and/or breed used;

(iii) Individual animal data for the following:

(A) Time of death during the study or whether animals survived to termination;

(B) Time of observation of each abnormal sign and its subsequent course;

(C) Food or water consumption, if appropriate;

(D) Body weight data;

(E) Results of ophthalmological examination, when performed;

(F) Hematological tests employed and results, with relevant baseline data, if available;

(G) Clinical biochemistry tests employed and results, with relevant baseline data, if available;

(H) Necropsy findings;

(I) Detailed description of all histopathological findings; and

(iv) Statistical treatment of results, where appropriate.

§ 83-2 Oncogenicity study.

(a) When required. Data from oncogenicity testing are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and end-use product that meet any of the following criteria:

(1) The active ingredient(s) or any of its (their) metabolites, degradation products, or impurities:

(i) Is structually related to a recognized carcinogen; or

(ii) Is a substance that causes mutagenic effect; or

(iii) Produces in subchronic studies a morphologic effect (e.g., hyperplasia, metaplasia) in any organ that may lead to neoplastic change;

(iv) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(2) The use requires a tolerance for the pesticide or exemption from the requirement to obtain a tolerance, or requires the issuance of a food additive regulation; or

(3) Use of the pesticide product is likely to result in human exposure over a portion of the human life span which is significant in terms of either the time the exposure occurs or the duration of exposure (for example: pesticides used in treated fabrics for wearing apparel, diapers, or bedding; insect repellents applied directly to human skin; pesticides applied to tobacco; swimming pool additives; constant-release indoor pesticides which are used in aerosol form).

(b) Purpose. The objective of a long-term carcinogenicity study is to observe test animals for a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration.

(c) Combined studies. For studies designed to meet the requirements of both the chronic toxicity studies and the oncogenicity study, refer to § 83-5.

(d) Substance to be tested. Testing shall be performed with the technical grade of each active ingredient in the product.

(e) Test procedures. (1) Animal selection. (i) Species and strain. It is recommended that a compound of unknown activity should be tested on two mammalian species. Rats and mice are the species of choice because of their relatively short life span, the limited cost of their maintenance, their widespread use in pharmacological and toxicological studies, their susceptibility to tumor induction, and the availability of inbred or sufficiently characterized strains. Commonly used laboratory strains should be employed. If other species are used, the tester should provide justification/reasoning for their selection.

(ii) Age. (A) Dosing of rodents should begin as soon as possible after weaning, ideally before the animals are six, but in any case not more than eight weeks old.

(B) At commencement of the study, the weight variation of animals should not exceed ± 20 percent of the mean weight for each sex.

(C) Studies using prenatal or neonatal animals may be recommended under special conditions.

(iii) Sex. (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. (A) For rodents, at least 100 animals (50 females and 50 males) should be used at each dose level and concurrent control.

(B) If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(C) The number of animals in any group should not fall below 50 percent at 15 months in mice and 18 months in rats at the termination of the experiment. At 18 month in mice and 24 months in rats the survival in any group should not fall below 25 percent.

(2) Control groups. (i) A concurrent control group is required. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended.

(ii) In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group is treated in the same manner as all other test animals, except that this control group should not be exposed to the test substance or any vehicle.

(3) Dose levels and dose selection. (i) For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group.

(ii) The highest dose level should be sufficiently high to elicit signs of minimal toxicity without substantially altering the normal life span.

(4) Exposure conditions. The animals are dosed with the test substance ideally on a 7-days-per-week basis over a period of at least 24 months for rats, and 18 months for mice. However, based primarily on practical considerations, dosing on a 5-days-per-week basis is considered to be acceptable.

(5) Observation period. It is necessary that the duration of an oncogenicity test comprise the majority of the normal life span of the strain of animals to be used. This time period should not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice. For longer time periods, and where any other species are used, consultation with the Agency in regard to the duration of the test is advised.

(6) Administration of the test substance. The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

(i) Oral studies. Provided it can be shown that the test substance is absorbed from the gastrointestinal tract, the oral route of administration is preferred.

(A) The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 24 months for rats and 18 months for mice.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(C) For a diet mixture, the highest concentration should generally not exceed 5 percent with the exception of nutrients.

(ii) Dermal studies. (A) The animals are treated by topical application with the test substance, ideally for at least 6 hours per day.

(B) The test substance should be applied uniformly over a shaved or clipped area which is approximately ten percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

(C) During the exposure period the test substance may be held in contact with the skin with a porous gauze dressing and nonirritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

(iii) Inhalation studies. (A) The animals are exposed by inhalation.

(B) The temperature at which the test is performed should be maintained at 22°C (± 2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols) this may not be practicable. Food and water should be withheld during exposure.

(C) A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(7) Observation of animals. (i) A careful clinical examination should be made at least twice each week.

(ii) Daily cageside observations should include, but not be limited to, changes in:

(A) Skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory;

(D) Circulatory;

(E) Autonomic and central nervous system;

(F) Somatomotor activity; and

(G) Behavior pattern.

(iii) Clinical signs and mortality should be recorded for all animals. Special attention should be paid to mass development. The following information on each grossly visible or palpable mass should be recorded:

(A) Time of onset;

(B) Location;

(C) Size;

(D) Appearance; and

(E) Progression.

(iv) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals, to ensure that not more than 10% of the animals in any test group are lost from the test due to cannibalism, autolysis of tissues, misplacement, and similar management problems.

(v) Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

(vi) Measurements of food or water consumption, respectively, should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

(vii) At the end of the study period all survivors are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(8) Clinical pathology. At 12 months, 18 months, and at sacrifice, a blood smear should be obtained from 10 animals/sex/dosage group animals. A differential blood count should be performed on blood smears from those animals in the highest dosage group and the controls. If these data, or data from the pathological examination indicate a need, then the 12 and 18 month blood smears from other dose groups should also be examined. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed.

(9) Gross necropsy. (i) A complete gross examination should be done in all animals, including those which died during the experiment or were killed in moribund conditions.

(ii) At least the liver, kidneys, brain, and testes should be weighed wet as soon as possible after dissection to avoid drying. For these organs, at least ten rodents per sex group should be weighed.

(iii) If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

(10) Tissue preservation. The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(A) Masses and associated tissues of all animals;

(B) All gross lesions of all animals;

(C) Brain - including sections of medulla/pons, cerebellar cortex, and cerebral cortex;

(D) Pituitary;

(E) Thyroid/parathyroid;

(F) Thymus;

(G) Trachea;

(H) Lungs;

(I) Heart;

(J) Salivary glands;

(K) Liver;

(L) Spleen;

(M) Kidneys;

(N) Adrenals;

(O) Pancreas;

(P) Testes;

(Q) Accessory genital organs, uterus;

(R) Female mammary gland;

(S) Skin;

(T) Esophagus;

(U) Stomach;

(V) Duodenum;

(W) Jejunum;

(X) Ileum;

(Y) Caecum;

(Z) Colon;

(AA) Rectum;

(BB) Urinary bladder;

(CC) Lymph nodes;

(DD) Peripheral nerve;

(EE) Spinal cord at three levels - cervical, midthoracic, and lumbar;

(FF) Sternum and/or femur with bone marrow;

(GG) Musculature;

(HH) Eyes;

(II) Gall bladder; and

(JJ) Aorta;

(ii) In special studies such as inhalation studies, the entire respiratory tract should be preserved, including nasal cavity, pharynx, and larynx.

(11) Histopathology. (i) The following histopathology should be performed:

(A) Full histopathology on organs and tissues, listed in paragraph (e)(1) of this section, of all animals in the control and high dose groups and all animals that died or were killed during the study;

(B) All gross lesions in all animals;

(B) All gross lesions in all animals; and

(C) Target organs in all animals.

(ii) The following histopathology should be performed:

(A) Lungs in all animals (special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals);

(B) Liver in all animals; and

(C) Kidneys in all animals.

(iii) If a significant difference is observed in hyperplastic, preneoplastic or neoplastic lesions between the highest dose and control groups, microscopic examination should be made on that particular organ or tissue of all animals in the study.

(iv) If excessive early death or other problems occur in the high dose group compromising the significance of the data, the next lower dose level shall be examined for complete histopathology.

(v) In case the results of the experiment give evidence for substantial alteration of the animals' normal longevity or the induction of effects that might affect a neoplastic response, the next lower dose level should be examined as described above.

(vi) An attempt should be made to correlate gross observations with microscopic findings.

(f) Data and reporting. (1) Treatment of results. (i) Data shall be summarized in tabular form, showing, for each test group:

(A) The number of animals at the start of the test;

(B) The number of animals showing lesions;

(C) The types of lesions; and

(D) The percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical method should be selected during the design of the study.

(2) Evaluation of study results. (A) The findings of an oncogenic toxicity study shall be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation will include the relationship

between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including:

(1) Behavioral abnormalities;

(2) Clinical abnormalities;

(3) Gross lesions;

(4) Identified target organs;

(5) Body weight changes;

(6) Effects on mortality; and

(7) Any other general or specific or toxic effects.

(B) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance shall be considered.

(3) Test report. In addition to information required by § 80-4, the test report summary should include the following information:

(i) Toxic response and other effects data by sex and dose;

(ii) Species and strain used; and

(iii) Individual animal data for the following:

(A) Time of death during the study or whether animals survived to termination;

(B) Time of observation of each mass and subsequent course;

(C) Food or water consumption, if appropriate;

(D) Body weight data;

(E) Hematological tests employed and results;

(F) Necropsy findings;

(G) Detailed description of all histopathological findings; and

(iv) Statistical treatment of results where appropriate.

§ 83-3 Teratogenicity study.

(a) When required. Data on teratogenicity studies are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and end-use product which meets either of the following criteria:

(1) Its pesticidal use, under widespread and commonly recognized practice, may reasonably be expected to result in significant exposure of acute duration to human females; or

(2) Its use requires a tolerance or an exemption from the requirement to obtain a tolerance, or its use requires issuance of a food additive regulation.

(3) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. The teratogenicity study is designed to determine the potential of the test substance to induce structural and/or other abnormalities to the fetus which may arise from exposure of the mother during pregnancy.

(c) Definitions. (1) "Teratogenicity" is the property of a chemical that causes permanent structural or functional abnormalities during the period of embryonic development.

(d) Standard of the test method. The test substance is administered in graduated doses, for at least that part of the pregnancy covering the period of organogenesis, to several groups of pregnant experimental animals, one dose level being used per group. Shortly before the expected date of delivery, the pregnant females are sacrificed, the uteri removed, and the contents examined for embryonic or fetal deaths, and live fetuses.

(e) Substance to be tested. Testing shall be performed with the technical grade of each active ingredient.

(f) Limit test. In the case of a substance of low toxicity, if a dose level of at least 1000 mg/kg produces no evidence of embryo toxicity or teratogenicity, studies at other dose levels may not be considered. If a preliminary study at this high dose level, with definite evidence of maternal toxicity, shows no adverse effects on embryos, studies at other dose levels may not be considered necessary. Test procedures should follow the guidelines of Section (g) below.

(g) Test procedures. (1) Animal selection. (i) Species and strain. Testing should be performed in at least two mammalian species. The preferred species are the rat and the rabbit. If other mammalian species are used, the tester should provide justification/reasoning for their selection. Commonly used laboratory strains should be employed. The strain should not have low fecundity and should preferably be characterized for its response to teratogens.

(ii) Age. Young adult animals should be used.

(iii) Sex. Pregnant animals should be used at each dose level.

(iv) Number. At least 20 pregnant rats, mice or hamsters or 12 pregnant rabbits are recommended at each dose level and control group. The objective is to ensure that sufficient pups are produced to permit meaningful evaluation of the teratogenic potential of the substance.

(2) Control group. A concurrent control group is recommended. This group should be an untreated or sham treated control group, or, if a vehicle of unknown toxicity is used in administering the test substance, a vehicle control group. Except for treatment with the test substance, animals in the control group(s) should be handled in an identical manner to test group animals.

(3) Dose levels and dose selection. (i) At least three dose levels with a control or, a vehicle control as indicated in (2) above should be used.

(ii) If a vehicle is used, its toxicological properties should be understood; it should not be teratogenic nor have effects on reproduction.

(iii) To select the appropriate dose levels, a pilot or trial study may be advisable. It is not always necessary to carry out a trial study in pregnant animals. Comparison of the results from a trial study in nonpregnant, and the main study in pregnant animals will demonstrate if the test substance is more toxic in pregnant animals.

(iv) Unless limited by the physical/chemical nature or biological properties of the substance, the highest dosage level should induce some overt maternal toxicity such as slight weight loss, but not more than 10 percent maternal deaths.

(v) The lowest dose level should not produce any evidence of toxicity.

(4) Observation period. Day 0 in the test is the day on which vaginal plug and/or sperm are observed (where feasible). The dose period should cover the period of major organogenesis. This may be taken as Days 6-15 for rat and mouse, 6-14 for hamster, or 6-18 for rabbit. If Day 0 is based on observation of mating or artificial insemination, the times stated should be adjusted by adding one day. Alternatively, the period of dosing may be extended to approximately one day before the expected delivery date.

(5) Administration of test substance. (i) The test substance or vehicle is usually administered orally, by oral intubation, unless the chemical or physical characteristics of the test substance or pattern of human exposure suggest a more appropriate route of administration.

(6) Exposure conditions. The female test animals are treated with the test substance daily throughout the appropriate treatment period. When given by gavage, the dose may be based on the weight of the females at the start of substance administration, or, alternatively, in view of the rapid weight gain which takes place during pregnancy, the animals may be weighed periodically and the dosage based on the most recent weight determination.

(7) Observation of animals. (i) A careful examination for clinical signs of toxicity should be made at least once each week to coincide with weighing (vi).

(ii) Additional observations should be made daily, with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead, and isolation or sacrifice of weak or moribund animals, to ensure that not more than 10% of the animals in any test group are lost from the test due to cannibalism, analysis of tissues, misplacement and similar management problems.

(iii) Signs of toxicity should be recorded as they are observed, including the time of onset, the degree, and the duration.

(iv) During the treatment and observation periods cageside observations should include, but not be limited to, changes in:

(A) Skin and fur;

(B) Eye and mucous membranes;

(C) Respiratory system;

(D) Circulatory system;

(E) Autonomic and central nervous system;

(G) Somatomotor activity; and

(H) Behavioral pattern.

(v) Measurements should be made weekly of food consumption for those animals in a dosed-feeding study.

(vi) Animals should be weighed weekly and at the time of sacrifice.

(vii) Females showing signs of abortion or premature delivery should be sacrificed and subjected to thorough macroscopic examination.

(8) Gross necropsy. (i) At the time of sacrifice or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

(ii) Immediately after sacrifice or death, the uterus should be removed and the contents examined for embryonic or fetal deaths and the number of viable fetuses. It is usually possible to estimate the time of death *in utero* where this has occurred.

(iii) The number of corpora lutea should be determined where possible.

(iv) The sex of the fetuses should be determined and each litter should be weighed, the weights recorded, and the mean fetal weight derived.

(v) Following removal, each fetus should be examined externally.

(vi) For rats, mice and hamsters, one third to one-half of each litter should be prepared and examined for skeletal anomalies, and the remaining part of each litter should be prepared and examined for soft tissue anomalies using appropriate methods.

(vii) For rabbits, each fetus should be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

(h) Data and reporting. (1) Treatment of results. (i) Data shall be summarized in tabular form, showing, for each test group:

(A) The number of animals at the start of the test;

(B) The number of pregnant animals;

(C) The number of corpora lutea and resorptions (early and late);

(D) The number and percentages of live fetuses for each dose group and in each litter; and

(E) The number of fetuses and litters containing fetuses with any soft tissue or skeletal abnormalities.

(2) Evaluation of results. The findings of a teratogenicity study shall be evaluated in terms of the observed effects and the dose levels producing effects. It is necessary to consider the historical teratogenicity data on the species/strain tested. Most properly conducted teratogenicity study should provide a satisfactory estimation of a no-effect level.

(3) Test report. In addition to the information required by § 80-4, the test report shall include the following information:

(i) Toxic response data by dose;

(ii) Species and strain;

(ii) Time of death during the study or whether animals survived to termination;

(iii) Time of observation of each abnormal sign and its subsequent course;

(iv) Food and body weight data;

(v) Pregnancy and litter data; and

(vi) Fetal data (live/dead, sex, soft tissue and skeletal defects, resorptions).

§ 83-4 Reproductive and fertility effects.

(a) When required. Data on a two-generation reproduction study are required by 40 CFR Part 158 to support the registration of each manufacturing-use product and formulated product that meets either of the following criteria:

(1) The pesticidal use of a pesticide product is likely to result in significant human exposure to the product, its active ingredient(s), metabolite(s), or degradation product(s); or

(2) The use requires a tolerance for the pesticide or an exemption from the requirement to obtain a tolerance, or requires issuance of a food additive regulation.

(3) See, specifically, 40 CFR § 158.50 and §158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. This guideline for two-generation reproduction testing is designed to provide general information concerning the effects of a test substance on gonadal function, estrus cycles, mating behavior, conception, parturition, lactation, weaning, and the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on teratogenesis and may serve as a guide for subsequent tests.

(c) Principle of the test method. The test substance is administered to parental (P_1) animals prior to their mating, during the resultant pregnancies, and through the weaning of their F_1 offspring. The substance is then administered to selected F_1 offspring during their growth into adulthood, mating, and production of an F_2 generation, up until the F_2 generation is 21 days old.

(d) Substance to be tested. Testing shall be performed with the technical grade of each active ingredient in the product.

(e) Test procedures: (1) Animal selection. (i) Species and strain. The rat or the mouse are the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection. Strains with low fecundity should not be used.

(ii) Age. Parental (P_1) animals should be about 8 weeks old at the start of dosing. See exposure conditions in paragraph (c)(4) of this section for dosing regimen.

(iii) Sex. (A) For an adequate assessment of fertility, both males and females must be studied.

(B) The females should be nulliparous and non-pregnant.

(iv) Each test and control group should contain at least 20 males and a sufficient number of females to yield at least 20 pregnant females at or near term.

(2) Control groups. (i) A concurrent control group is recommended. This group should be an untreated or sham treated control group or if a vehicle is used in administering the test substance, a vehicle control group.

(ii) If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used.

(iii) If a vehicle or other additive is used to facilitate dosing, it should not interfere with absorption of the test substance or produce toxic effects.

(3) Dose levels and dose selection. (i) At least three dose levels and a concurrent control should be used.

(ii) The highest dose level should induce toxicity but not mortality in the parental (P_1) animals.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is usable estimation of human exposure the lowest dose should exceed this.

(iv) The intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, dose levels should be spaced to produce a gradation of toxic effects.

(v) The incidence of fatalities in low and intermediate dose groups and in the controls should be low, to permit meaningful evaluation of the results.

(4) Exposure conditions. The animals are dosed with the test substance ideally on a seven-days per week basis using the testing schedule presented in Table 3.

(i) Table 4 contains the dosing, mating, delivery, and sacrifice schedule for animals on test.

(A) Daily dosing of the parental (P_1) males and females should begin when they are about 8 weeks old. For both sexes, dosing should be continued for at least eight weeks before the mating period.

(B) Dosing of P_1 males should continue through the mating period. At the end of the mating period, P_1 males should be sacrificed and examined. Dosing of the F_1 males saved for mating should continue from the time they are weaned through the period they are mated with the F_1 females (11 weeks for mice and 17 weeks for rats). F_1 males may be sacrificed after the F_1 mating period.

(C) Daily dosing of the P_1 females should continue through the three week mating period, pregnancy, and to the time of weaning three weeks after delivery. Dosing of the F_1 females saved for mating should continue from the time they are weaned, through the period they are mated with the F_1 males (11 weeks for mice and 17 weeks for rats), through pregnancy, and to the weaning of the F_2 offspring.

(ii) All animals are sacrificed as follows:

(A) All P_1 males should be sacrificed immediately after delivery of the litter last sired or, in cases of infertility, after the conditions of (7)(i)(B) are met.

(B) F_1 males selected for mating should be sacrificed immediately after delivery of the last F_1 litter sired, or, in cases of infertility, after the conditions of (7)(i)(B) are met.

(C) F_1 males and females not selected for mating should be sacrificed when weaned.

(D) The parental females in all generations should be sacrificed upon weaning of their last litters.

(E) F_2 offspring are sacrificed when the offspring are 21 days of age.

(5) Observation period. Duration of observation should be for at least 28 weeks from dosing of P_1 animals to sacrifice of F_2 offspring at weaning.

(6) Administration of the test substance. The oral route of administration is preferred.

TABLE 4. APPROXIMATE DOSING AND BREEDING SCHEDULE

Weeks on Study*	P_1	F_1	F_2
0	Dosing of P_1 males and females begins		
8-14	P_1 mating period		
11-17	Dosing of P_1 males may end at Week 25; P_1 males are sacrificed *	F_1 born and litter sizes randomly adjusted to 8 pups each	
14-20	Dosing of P_1 females ends P_1 females are sacrificed	F_1 weaned; dosing of F_1 females begins F_1 offspring not selected for mating are sacrificed	
22-34		F_1 mating; Dosing of F_1 males may end at Week 40; F_1 males are sacrificed**	
25-37		Remaining F_1 females are sacrificed	F_2 born and litter sizes randomly adjusted to to 8 pups each F_2 offspring are sacrficed

*The lower number in the ranges is considered appropriate for mice, and the upper number is appropriate for studies using rats. Earliest breeding age for mice is approximately 8 weeks and that for rats is 14 weeks. This schedule is for studies using one mating per generation. Numbers should be adjusted by appropriate numbers of weeks to accommodate the second mating, gestation and weaning of offspring as well as rest period between matings.

**This is appropriate for studies with two matings per generation or the time may be longer in order that paragraph (7)(i)(A) can be satisfied.

(i) When administered by gavage or capsule, the dosage administered to each animal prior to mating should be based on the individual animal's body weight and adjusted weekly. During pregnancy the dosage should be based on the body weight at Day 0 and 6 of pregnancy.

(ii) It is recommended that the test substance be administered in the diet or drinking water.

(iii) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(iv) For a diet mixture, the highest concentration should not exceed five percent with the exception of nutrients.

(7) Mating procedure. (i) Parental. (A) For each mating, each female should be placed with a single randomly selected male from the same dose level until pregnancy occurs or three weeks have elapsed. Paired matings should be clearly identified and mixed matings with other males avoided.

(B) Those pairs that fail to mate successfully should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with other sires or dams, histological examination of the reproductive organs, and examination of the estrus or spermatogenic cycles.

(C) Each morning the female should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day vaginal plugs or sperm are found.

(ii) F_1 cross. (A) For mating the F_1 offspring, one male and one female are randomly selected from each litter for cross mating to produce the F_2 generation. Mating of siblings should be avoided.

(B) F_1 males and females not selected for mating are sacrificed upon weaning.

(iii) Special housing. Near parturition, pregnant animals should be caged separately in delivery or maternity cages and provided with nesting materials.

(iv) Standardization of litter sizes. (A) On Day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, at least four males and four females per litter.

(B) Whenever the number of male or female pups prevents having at least four of each sex per litter, partial adjustment (for example, five males and three females) is permitted. Adjustments are not appropriate for litters of less than eight pups.

(C) Adjustments of the F_2 litters is conducted in the same manner.

(8) Observation of animals. (i) A careful examination for clinical signs of toxicity should be made at least once each day. Pertinent behavioral changes, signs of difficult or prolonged parturition, food consumption and all signs of toxicity, including mortality, should be recorded. These observations should be reported for each individual animal.

(ii) The duration of gestation should be calculated from Day 0 of pregnancy.

(iii) Each litter should be examined as soon as possible after delivery for the number of pups, stillbirths, live births, and the presence of gross anomalies. Dead pups and pups sacrificed at Day 4 should be preserved and studied for possible defects and cause of death. Live pups should be counted and litters weighed, by weighing each individual pup (optional) at birth, or soon thereafter, and on Days 4, 7 (optional), 14, and 21 after birth.

(iv) Physical or behavioral abnormalities observed in the dams or offspring should be recorded.

(v) P_1 males and females should be weighed on the first day of dosing and weekly thereafter. F_1 litters should be weighed at birth, or soon thereafter, and on Days 4, 7, (optional) 14, and 21. Those F_1 offspring selected to produce F_2 litters should be weighed at birth, or soon thereafter, and on days 4, 7 (optional), 14, and 21 after birth. Individual pup weights should be measured at day 21.

(9) Gross necropsy. (i) A complete gross examination should be done on all animals, including those which died during the experiment or were killed in moribund conditions, "to insure that not more than 10% of the animals in any test group are lost from the test due to cannibalism, analysis of tissues, misplacement and similar management problems."

(ii) Special attention will be directed to the organs of the reproductive system.

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(iii) The following organs and tissues, or representative samples:

(A) Vagina;

(B) Uterus;

(C) Ovaries;

(D) Testes;

(E) Epididymus;

(F) Seminal vesicles;

(G) Prostate; and

(H) Target organ(s) of all P_1 and F_1 animals selected for mating.

(10) Histopathology. (i) The following histopathology should be performed:

(A) Full histopathology on the organs listed in paragraph (e)(9)(iii) of this section for all high dose and control P_1 and F_1 animals selected for mating.

(B) Organs demonstrating pathology in these animals should then be examined in animals from the other dose groups.

(C) Microscopic examination should be made of all tissues showing gross pathological changes.

(f) Data and reporting. (1) Treatment of results. (i) Data shall be summarized in tabular form, showing, for each test group:

(A) The number of animals at the start of the test;

(B) The number of animals pregnant;

(C) The types of change (see sections 83-4(e)(8),(9), and (10); and

(D) The percentage of animals displaying each type of change.

(2) Evaluation of study results. An evaluation of test results, including the statistical analysis, based on the clinical findings, the gross necropsy findings, and the microscopic results, shall be made and supplied. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities.

(3) Test report. In addition to the information required by § 80-4, the test report shall include the following information:

(i) Toxic response data by sex and dose, including fertility indices and length of gestation;

(ii) Species and strain;

(iii) Time of death during the study or whether animals survived to termination;

(iv) Toxic or other effects on reproduction, offspring, or postnatal growth;

(v) Time of observation of each abnormal sign and its subsequent course;

(vi) Body weight data for P_1, F_1 and F_2 animals;

(vii) Necropsy findings;

(viii) Detailed description of all histopathological findings; and

(ix) Statistical treatment of results where appropriate.

§ 83-5 Combined chronic toxicity/oncogenicity studies.

(a) Purpose. The objective of a combined chronic toxicity/carcinogenicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. The application of these standards should generate data on which to identify the majority of chronic and oncogenic effects and to determine dose-response relationships. Ideally, the design and conduct should allow for the detection of neoplastic effects and a determination of oncogenic potential as well as general toxicity, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

(b) Test procedures. (1) Animal selection. (i) Species and strain. Preliminary studies providing data on acute, subchronic, and toxicokinetic responses should have been carried out to permit an appropriate choice of animals (species and strain). As discussed in other guidelines, the mouse and rat have been most widely used for assessment of carcinogenic potential, while the rat and dog have been most often studied for chronic toxicity. The rat is the species of choice for combined chronic toxicity and carcinogenicity studies. If other species are used, the tester should provide justification/reasoning for their selection. Where available, the strain selected should be susceptible to the carcinogenic or toxic effect of the class of substances being tested if known, and provided it does not have a spontaneous background too high for meaningful assessment. Commonly used laboratory strains should be employed.

(ii) Age. (A) Dosing of rats should begin as soon as possible after weaning, ideally before the rats are six, but in any case not more than eight weeks old.

(B) At commencement of the study, the weight variation of animals should not exceed ± 20 percent of the mean weight for each sex.

(C) Studies using prenatal or neonatal animals may be recommended under special conditions.

(iii) Sex. (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) Numbers. (A) For rats, at least 100 animals (50 females and 50 males) should be used at each dose level and concurrent control.

(B) If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(C) The number of animals in any group should not fall below 50 percent at 15 months in mice and 18 months in rats. At the termination of the experiment at 18 months in mice and 24 months in rats the survival in any groups should not fall below 25 percent.

(2) Control groups. (i) A concurrent control group (50 females and 50 males) should be utilized. These groups should be untreated or sham treated control groups or, if a vehicle is used in administering the test substance, vehicle control groups. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are to be used. Animals (10/sex) in the satellite control group should be sacrificed at the same time the satellite test group is terminated.

(3) Dose levels and dose selection. (i) For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group.

(ii) The highest dose level in rodents should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results.

(vi) For chronic toxicological assessment, an additional treated and a concurrent control satellite group may be included in the study. The highest dose for satellite animals should be chosen so as to produce frank toxicity, but not excessive lethality, in order to elucidate a toxicological profile of the test substance.

(4) Exposure conditions. Ideally the animals are dosed daily with the test substance on a 7-days-per-week basis over a period of at least 24 months for rats and 18 months for mice and hamsters. The satellite animals are dosed as above for at least a 12 month period, for a non-food use and at least 24 months for a food use chemical.

(5) Observation period. It is necessary that the duration of the oncogenicity test comprise the majority of the normal life span of the animals to be used. It has been suggested that the duration of the study should be for the entire lifetime of all animals. However, a few animals may greatly exceed the average lifetime and the duration of the study may be unnecessarily extended and complicate the conduct and evaluation of the study. Rather, a finite period covering the majority of the expected life span of the strain is preferred since the probability is high that, for the great majority of chemicals, induced tumors will occur within such an observation period. The following guidelines are recommended:

(i) Generally, the termination of the study should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumor rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. For longer time periods, and where any other species are used, consultation with the Agency in regard to duration of the test is advised.

(ii) When included, the satellite groups and the concurrent satellite control group should be retained in the study for at least 12 months for a non-food use and at least 24 months for food use chemicals. These animals should be scheduled for sacrifice for an estimation of test-substance-related pathology uncomplicated by geriatric changes.

(6) Administration of the test substance. The three main routes of administration are oral, dermal, and inhalation. The choice of the route of adminstration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans. The oral route of administration is preferred.

(i) For studies using oral administration, the following procedures should be followed:

(A) The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 24 months for rats and 18 months for mice and hamsters.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(C) For a diet mixture, the highest concentration should not exceed five percent, with the exception of nutrients.

(7) Observation of animals. (i) A careful clinical examination should be made at least once each week.

(ii) Daily cageside observations should include, but not be limited to, changes in:

(A) Skin and fur;

(B) Eyes and mucous membranes;

(C) Respiratory;

(D) Circulatory;

(E) Autonomic and central nervous system;

(F) Somatomotor activity; and

(G) Behavior pattern.

(iii) Clinical signs of toxicity including suspected tumors and mortality should be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals, to insure that not more than 10% of the animals in any test group are lost from the test due to cannibalism, autolysis of tissues, misplacement, and similar management problems.

(i) Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

(vi) Measurements of food or water consumption should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

(vii) At the end of the study period all survivors are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(8) Clinical pathology. (i) The following examinations should be made on at least 10 rodents of each sex per dose level:

(A) Certain hematology determinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at approximately six month intervals during the conduct of the study and at study termination, on blood samples collected from 10 rodents/sex of all groups. If possible these collections should be from the same animals at each interval. Differential white blood cell counts of control and highest dose animals, and only if necessary for the intermediate dose animals, should be determined at the same intervals. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential leukocyte count should be performed on samples from those animals in the highest dosage group and the controls. Differential leukocyte counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls.

(B) Certain clinical biochemistry determinations on blood should be carried out at approximately 6-month intervals and at termination. Blood samples should be drawn for clinical chemistry measurements from at least ten rodents per sex of all groups and, if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests should be influenced by observations on the mode of action of the substance. Suggested determinations are:

(1) Calcium;

(2) Phosphorus;

(3) Chloride;

(4) Sodium;

(5) Potassium;

(6) Fasting glucose (with period of fasting appropriate to the species);

(7) Serum glutamic-pyruvic transaminase (also known as serum alanine aminotransferase);

(8) Serum glutamic-oxaloacetic transaminase (also known as serum aspartite aminotransferase);

(9) Blood urea nitrogen;

(10) Albumen;

(11) Creatine phosphokinase;

(12) Total cholesterol;

(13) Total bilirubin; and

(14) Total serum protein measurements.

(15) Other determinations which may be necessary for an adequate toxicological evalution include analyses of lipids, hormones, acid/base balance, methemoglobin, and cholinesterase activity.

(16) Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(ii) The following should be performed on at least ten rodents of each sex per dose level:

(A) Urine samples, if possible from the same rodents at the same intervals as hematological examination above, should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents:

(1) Appearance, volume, and specific gravity, for individual animals;

(2) Protein;

(3) Glucose;

(4) Ketones;

(5) Occult blood (semi-quantitatively); and

(6) Microscopy of sediment (semi-quantitatively).

(B) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at termination of the study, preferably in all animals, but at least in the high dose and control groups. If changes in the eyes are detected all animals should be examined.

(9) Gross necropsy. (i) A complete gross examination should be done in all animals, including those which died during the experiment or were killed in moribund conditions.

(ii) At least the liver, kidneys, brain, and testes should be weighed wet as soon as possible after dissection to avoid drying. For these organs, at least ten rodents per sex per group should be weighed.

(iii) If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

(10) Tissue preservation. (i) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(A) All gross lesions and tumors;

(B) Spinal cord at three levels - cervical, midthoracic, and lumbar;

(C) Brain - including sections of medulla/pons, cerebellar cortex and cerebral cortex;

(D) Pituitary;

(E) Thyroid/parathyroid;

(F) Thymus;

(G) Trachea;

(H) Lungs;

(I) Heart;

(J) Salivary glands;

(K) Liver;

(L) Spleen;

(M) Kidneys;

(N) Adrenals;

(O) Pancreas;

(P) Testes;

(Q) Accessory genital organs, uterus;

(R) Female mammary gland;

(S) Gall bladder (if present);

(T) Esophagus;

(U) Stomach;

(V) Duodenum;

(W) Jejunum;

(X) Ileum;

(Y) Caecum;

(Z) Colon;

(AA) Rectum;

(BB) Urinary bladder;

(CC) Skin;

(CC) Lymph nodes;

(DD) Peripheral nerve;

(EE) Sternum and/or femur with bone marrow;

(FF) Musculature;

(GG) Eyes; and

(HH) Aorta.

(ii) In special studies, such as inhalation studies, the entire respiratory tract should be preserved including nose, pharynx, and larynx.

(iii) Although inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues, the inflation of the lungs only in inhalation studies is essential for appropriate histopathological examination.

(11) Histopathology. (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed in paragraph (b)(11) of this section, of all animals in the control and high dose groups and all animals that died or were killed during the study;

(B) All gross lesions in all animals; and

(C) Target organs in all animals.

(ii) The following histopathology should be performed:

(A) Lungs of all animals (special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals);

(B) Livers of all animals; and

(C) Kidneys of all animals.

(iii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iv) In case the result of the experiment gives evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic response, the next lower dose level should be examined as described above.

(v) An attempt should be made to correlate gross observations with microscopic findings.

(c) Data and reporting. (1) Treatment of results. (i) Data shall be summarized in tabular form, showing for each test group:

(A) The number of animals at the start of the test;

(B) The number of animals showing lesions;

(C) The types of lesions; and

(D) The percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical method should be selected during the design of the study.

(2) Evaluation of study results. (A) The findings of a combined chronic toxicity/oncogenicity study shall be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation should include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including:

(1) Behavioral abnormalities;

(2) Clinical abnormalities;

(3) Gross lesions;

(4) Identified target organs;

(5) Body weight changes;

(6) Effects on mortality; and

(7) Any other general or specific or chronic toxic effects.

(B) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered. In order for a negative test to be acceptable, it should meet the following criteria:

(3) Test report. In addition to information required by § 80-4, the test report summary shall include the following information:

(i) Toxic response and other effects data by sex and dose;

(ii) Species and strain used;

(iii) Individual animal data for the following:

(A) Time of death during the study or whether animals survived to termination;

(B) Time of observation of each abnormal sign and its subsequent course;

(C) Food or water consumption;

(D) Body weight data;

(E) Results of ophthalmological examination, when performed;

(F) Hematological tests employed and results, with relevant baseline data, if available;

(G) Clinical biochemistry tests employed and results, with relevant baseline data, if available;

(H) Necropsy findings;

(I) Detailed description of all histopathological findings; and

(iv) Statistical treatment of results where appropriate.

Series 84: Mutagenicity.

84-1 Purpose and General Recommendations for Mutagenicity Testing.

(a) When required. As required by 40 CFR § 158.135 mutagenicity data shall be submitted to support the registration of each manufacturing-use product and end-use product that meet any of the following criteria:

(1) The use requires a tolerance for the pesticide or exemption from the requirement to obtain a tolerance, or require the issuance of a food additive regulation; or

(2) The pesticide product is likely to result in significant human exposure; or

(3) The active ingredient(s) or any of its (their) metabolites is structurally-related to a mutagen or oncogen, or belongs to any chemical class of compounds containing a significant number of mutagens or oncogens.

(4) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. For each test substance a battery of tests is required to assess the potential to affect the qualitative or quantitative integrity of the mammalian cell's genetic components. The objectives underlying the selection of a battery of tests for mutagenicity assessment are:

(1) To detect, with sensitive assay methods, the capacity of a chemical to alter genetic material in cells;

(2) To determine the relevance of these mutagenic changes to mammals, and when mutagenic potential is demonstrated;

(3) To incorporate these findings in the assessment of heritable effects, oncogenicity, and possibly, other health endpoints.

(c) Substance to be tested. Testing shall be performed with the technical grade of each active ingredient in the product.

(d) Standards for metabolic activation. (1) Chemicals are often non-mutagenic unless converted to an active mutagen by metabolic processing. The reverse can also occur. Therefore, a metabolic activation system should be incorporated into any test system other than intact mammals and insects.

(2) The test substance should be tested both in the presence and the absence of mammalian tissue extracts (with appropriate cofactors) which have been demonstrated to convert a wide range of chemical "promutagens"

(substances which are mutagenically-inactive in the absence of the tissue extracts) to mutagenically-active substances. Rat liver extracts are preferred. The tissue should be pre-induced for the relevant enzymatic activities when appropriate. The inducer should be effective for the class of compounds under test. Other tissue extracts should be used in addition to liver extracts when the principal site of metabolism of the test substance is known not to be the liver, or when other tissues, including plant tissue, are known to give positive results with chemicals structurally-related to these chemicals.

(3) The test substance may also be exposed to metabolic processing in intact mammals by a host-mediated system in which the target cells are inserted into host tissues or body cavities. Hepatocytes may also be used to provide metabolic processing, either as a co-culture with a target cell, or as the primary assay system.

(e) Controls. All assays should be run with concurrent positive and negative controls with the possible exception of the mouse specific locus test.

(1) Positive controls. Positive control compounds should be selected to demonstrate both the sensitivity of the indicator organism and the functioning of the metabolic activation system. Positive controls should also be selected to demonstrate the sensitivity of the indicator cells or organisms to a compound with chemical characteristics similar to those of the test substance. For instance, an alkylating agent should be used as a control for an expected alkylator, and an intercalating agent for a suspected intercalator. Where applicable, the positive control should be administered by the same route as the test substance.

(2) Negative controls. Both a solvent and where applicable, a nonsolvent negative control, should also be included.

84-2 Mutagenicity Tests.

(a) Tests required. The battery must include tests appropriate to address the following three categories in accordance with the purposes (84-1(b)):

(1) Gene mutations;
(2) Structural chromosomal aberrations; and
(3) Other genotoxic effects as appropriate for the test substance, e.g., numerical chromosome aberations, direct DNA damage and repair.

(b) Representative tests. A representative selection of tests within each category follows. The most commonly used organism, cell type, or animal is indicated; others may be acceptable if sufficient testing is done to verify their usefulness.

(1) Gene mutation tests.

(i) Microorganisms.

(A) Bacteria, reverse mutation:

Salmonella typhimurium, Ames' strains
Escherichia coli, WP2 and WP2 uvrA
Bacillus subtilis TKJ 5211, TKJ 6321

(B) Eucaryotic microorganisms, forward and reverse mutations:

Saccharomyces cerevisiae
Schizosaccharomyces pombe
Neurospora crassa
Aspergillus nidulans

(ii) Submammalian organisms, sex-linked recessive lethal:

Drosophila melanogaster

(iii) Mammalian cells in culture, forward or reverse mutations at specific foci:

Chinese hamster lung (V79)
Chinese hamster ovary (CHO)
Mouse Lymphoma (L5178Y)

(iv) Specific locus:

Mouse

(2) Structural chromosome aberration tests.

(i) Eucaryotic microorganisms:

Aspergillus nidulans
Neurospora crassa

(ii) Submammalian organisms, chromosome tests:

Drosophila melanogaster

(iii) Mammalian cells in culture:

(A) Sister chromatid exchange

(B) Cytogenetic analysis

(iv) Mammals:

(A) Micronucleus test

- (B) Sister chromatid exchange
- (C) Cytogenetic analysis
- (D) Dominant lethal:
  - Mouse
  - Rat
- (E) Heritable translocation:
  - Mouse

(3) Tests for other genotoxic effects.

(i) DNA damage and repair.

- (A) Differential toxicity in bacteria:
  - *Escherichia coli* pol A+/pol A-
  - *Bacillus subtilis* H17/M45
- (B) Mitotic recombination in eucaryotic organisms:
  - *Saccharomyces cerevisiae*
  - *Aspergillus nidulans*
- (C) Unscheduled DNA synthesis:
  - Mammalian cells in culture
  - Mouse
- (D) DNA alkaline elution:
  - Cells in culture
- (E) Sister chromatid exchange:
  - Cells in culture
  - Mammals

(ii) Numerical chromosomal aberrations.

- (A) Eucaryotic microorganisms, mitotic segregation.
- (B) Mitotic interference:
  - Cells in culture
  - Mammals

(C) Micronucleus test:

Cells in culture
Mammals

(iii) Mammalian cell transformation:

Cells in culture

(iv) Target organ/cell analysis:

(A) Sperm morphology

(B) DNA synthesis inhibition

(C) DNA alkylation

(c) Current standards for test protocols, conduct of study and presentation of data are found in publications from the Gene-Tox Program of the EPA Office of Toxic Substances (appearing in Mutation Research) and the EPA/SRI International Project "In Vitro Mutagenicity Studies of Environmental Chemicals," 1982. Improvements in state of the art criteria will be listed by EPA in the NTIS.

Because of the rapid improvements in this field, registrants are encouraged to discuss with the Agency testing battery selection, protocol design, and results of preliminary testing.

Series 85: SPECIAL STUDIES

[NOTE: Section 85-1 of this series is prepared in conformity with the guidelines developed by the Organization of Economic Cooperation and Development. Those guidelines were adapted to fit the toxicology data requirements of this section under FIFRA.

Section 85-2 is essentially identical to § 163.86-1 of the Subpart F guidelines proposed on August 22, 1978 (43 FR 37336). Public comments on this section have not yet been used to revise the section. When the revision is prepared, it will replace the current § 85-2.]

§ 85-1 Metabolism study.

(a) When required. Data from a general metabolism study are required to support the registration of each manufacturing-use product and each end-use product which requires a chronic toxicity study or an oncogenicity study, in accordance with 40 CFR § 158.135.

(1) See, specifically, 40 CFR § 158.50 and § 158.135 to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the "Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. (1) Data from studies on the absorption, distribution, excretion, and metabolism of a test chemical are desirable to aid in the evaluation of test results from other toxicology studies and in the extrapolation of data from animals to man. Such studies should ideally be done on each chemical of toxicological concern. The concern may be predicated on the level and type of toxicity observed (or anticipated) and by the magnitude of potential human exposure to the chemical. Flexibility is needed in the conduct of metabolism studies and depends on the characteristics of the test chemical being investigated. The main purpose of metabolism studies is to produce data which fortify the understanding of the safety of the chemical in consideration of its intended uses and anticipated human exposure.

(2) In addition to the general reasons stated in paragraph (b)(1) of this section, a metabolism study may be performed for the following purposes:

(i) To determine the amount and rate of absorption of the test chemical at different dose levels;

(ii) To determine the pattern of distribution of the test chemical among tissues, organs, and fluid compartments at different dose levels, after single and repeated dosages;

(iii) To identify and, to the extent possible, quantify significant metabolites;

(iv) To characterize route(s) and rate(s) of excretion;

(v) To determine any possible bioaccumulation and/or bioretention of the test substance and/or metabolites; and

(vi) To determine absorption, metabolism, excretion, and distribution as a function of single or repeated doses. For certain chemicals, metabolism studies may not adequately define all of these processes.

(c) Labeled test material. (1) Single-dose testing shall be performed with an analytically pure grade of the active ingredient, usually in an isotopically-labeled form. The label may be radioactive, such as ^{14}C, ^{35}S, and ^{36}Cl, or stable, such as ^{15}N and ^{18}O. In some cases, more than one label per molecule may be advantageous. Labels shall be placed in positions that may be expected to follow the "core" of the molecule or significant portions thereof. If possible, one should avoid placing ^{14}C in positions from which it may be expected to enter the carbon pool of the test animal. Use of readily exchangeable labeling shall be avoided.

(2) Labeled compound may not be required if sufficiently selective and sensitive physical-chemical tests for identifying the compound and its metabolites are used.

(3) Some animals are to receive repetitive doses of nonlabeled chemical substance (analytical grade).

(d) Test procedure. (1) Choice of method. A registrant may, after consultation with the Agency, utilize a modified or completely different experimental design if it provides the information required by this section.

(2) Animal selection. (i) Species and strain. The preferred species is the rat. If another mammalian species is used, the tester should provide justification/reasoning for its selection. Commonly-used laboratory strains should be employed. Preliminary studies may be performed in several species to develop information on comparative metabolism. Information derived from preliminary studies may help in the selection of species for subsequent toxicity tests.

(ii) Age. Young adult animals should be used. For specific purposes, a comparative study using very young or very old animals may provide information about the effects of age on the metabolism.

(iii) Sex. (A) Equal numbers of animals of each sex should be used at each dose level.

(B) Females should be nulliparous and nonpregnant.

(iv) Numbers. At least ten animals (five females and five males) should be used at each dose level.

(3) Dose levels and dose selection. (i) At least two dose levels should be used.

(ii) The low dose should correspond to a no-effect level.

(iii) The upper dose should produce toxic or pharmacologic signs, but should not produce severe effects or a high incidence of mortality which would prevent a meaningful evaluation.

(iv) The determination of absorption, tissue distribution, and elimination should be studied as a function of single or repeated doses.

(4) Observation period. Animals should be kept in individual metabolism cages for 7 days after radioactive dose or until 90+ percent of the administered dose is excreted (whichever occurs first), at which time all of the animals should be killed.

(5) Administration of the test substance. (i) Route of administration. The study should be done using the oral route (capsule or gavage). If another route of administration is used, the tester should provide justification/reasoning for its selection. When vehicles are used, attention must be given to the possibility that they may interfere with the kinetics of the test chemical.

(ii) Animal groups. The following four groups of animals should be studied:

(A) Group A. These animals should each receive a single intravenous dose of the labeled test substance at the low dose. If it is not possible to dissolve the test substance in physiological saline or water, this group may be omitted.

(B) Group B. These animals should each receive a single oral dose (by capsule or intubation) of the labeled test substance at the low dose.

(C) Group C. These animals should each receive a series of single daily oral doses of the nonlabeled test substance (by capsule or intubation) over a period of least 14 days, followed at 24 hours after the last dose by a single oral dose (by capsule or intubation) of the labeled test substance. Each dose should be the low dose level.

(D) Group D. These animals should each receive a single oral dose (by capsule or intubation) of the labeled test substance at the high dose level.

(6) Observations of animals. (i) Distribution. For all animals in Groups B, C, and D, the quantity of label in tissues and organs should be measured at sacrifice by suitable methods with particular attention to bone, brain, fat, gonads, heart, kidney, liver, lungs, muscle, spleen, tissues which displayed pathology (in this or prior studies), and residual carcass.

(ii) Metabolism. Urine and feces from all groups should be analyzed by suitable methods in order to determine the extent of absorption and biotransformation and to identify the metabolites. An assay method for detection of each major metabolite may be requested by the Agency.

(iii) Excretion. Quantities of label in urine, feces, and expired air should be measured at appropriate intervals (e.g., 4, 8, 12, and 24 hours, 1.5, 2, 3, 4, 5, 6, and 7 days) throughout the study for all animals. However, if a preliminary study shows no volatile label materials are exhaled during the period of zero to 24 hours after dosing, such evidence may be submitted in lieu of measuring label in the expired air for this study.

(e) Data and reporting.

(1) Treatment of results. Data shall be summarized in tabular form.

(2) Evaluation of results. Results, where appropriate, shall be evaluated statistically.

(3) Test report. In addition to the information required by § 80-4, the test report shall include the following data derived from tests on animals in all groups:

(i) Quantity of isotype, together with percent recovery of the administered dose, in feces, urine, and the following tissues and organs of animals in all groups:

(A) Bone;

(B) Brain;

(C) Fat;

(D) Testes;

(E) Heart;

(F) Kidney;

(G) Liver;

(H) Lung;

(I) Blood;

(J) Muscle;

(K) Spleen;

(L) Tissues which displayed pathology (in this or prior studies);

(M) Literus; and

(N) Residual carcass.

(ii) Percent absorption; if possible, percent absorption by the oral route in Groups B, C, and D;

(iii) A full description of the sensitivity and precision of all procedures used to produce the data;

(iv) Information on the degree (i.e., specific activity for a radiolabel) and site(s) of labeling of the test substance; and

(v) Counting efficacy data; such data should be recommended, however, only upon specific request of the Agency.

(f) Additional metabolism studies. Additional, more specific studies may be required to clarify important points.

(1) Some areas for possible further study include:

(i) Identification of tissue residues;

(ii) Binding by macromolecules in the blood, liver, gonads, and other tissues; plasma binding studies may be conducted, usually *in vitro* with plasma;

(iii) Placental transfer; placental transfer of a chemical substance may be determined by dosing pregnant rodents with chemicals and assaying their fetuses for the chemical;

(iv) Entrance into breast milk;

(v) Biotransformation by specific organs, tissues, and cell fractions; and

(vi) Absorption by dermal or inhalation routes of exposure.

(2) Additional species may be utilized, since the rat and dog differ significantly in metabolic pattern.

§ 85-2 Domestic animal safety testing.

(a) When required. Data from tests on domestic animals may be required in accordance with 40 CFR § 158.135 to support the registration of an end-use product if cats, dogs, cattle, pigs, sheep, or other domesticated animals will be exposed to the pesticide product, including, but not limited to, exposure through direct application for pest control and consumption of treated feed. The applicant for registration should consult with the Agency to determine what toxicological data are required. In some cases, the data resulting from studies performed on laboratory and nondomestic animals can be extrapolated to the domestic species likely to be exposed. In these cases, no additional testing will be required.

(b) Testing. Data from any of the studies described in this subdivision may be required, including, but not limited to, the following:

(1) Acute oral toxicity;

(2) Acute dermal toxicity;

(3) Acute inhalation toxicity;

(4) Primary dermal irritation;

(5) Primary eye irritation;

(6) Dermal sensitization;

(7) Subchronic oral dosing;

(8) Cholinesterase inhibition;

(9) Neurotoxicity; and

(10) Teratogenicity.

(c) Standards. Each test should be performed according to the standards specified by the Agency. The applicant should also refer to standards specified in the appropriate sections of this subdivision.

(d) Data reporting and evaluation. (1) The general information required by § 80-4 shall be reported for each test. In addition, each test report shall contain all appropriate data required by the "Data reporting and evaluation" paragraphs of the corresponding sections of this subdivision.

(2) In addition, the applicant should submit any evidence of toxicological effects of the pesticide to domestic animals observed during product performance testing including, in particular, field testing.

§ 85-3 Dermal Absorption Studies of Pesticides. [Reserved]